Inherited disorders of galactose metabolism and cultured cells

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INHERITED DISORDERS of GALACTOSE

METABOLISM and CULTURED CELLS

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SUMMARY

The inherited disease galactosaemia is caused by a deficiency of galactose-1-phosphate uridyl transferase. A related clinically serious disorder caused by a deficiency of uridine diphosphate galactose 4′ epimerase has now been found and an animal model produced in CHO cells. Completely epimerase deficient cells would be unable to synthesise the galactose required for important structural components.

This thesis investigates the effect on cultured cells of these disorders of galactose metabolism, including whether epimerase deficient cells need galactose supplementation for normal growth.

Four classes of experiment were carried out:

1) **Galactose content of culture media.** It was necessary to establish rigorously the conditions in which cells were cultured. Analysis showed no measurable free galactose in the culture medium, but the presence of glycoproteins with exposed galactosyl residues was demonstrated.

2) **Cell growth in normal media.** Epimerase deficient fibroblasts and CHO cells did not require galactose supplementation for growth. Transferase deficient cells took up significantly more uridine and thymidine, demonstrating important abnormalities in purine and pyrimidine metabolism.

3) **Growth in media supplemented with galactose.** Control cell lines increased in number, transferase deficient lines initially increased then decreased, showing a delayed toxic effect, and epimerase deficient cells steadily decreased.

4) **Galactose content of cells.** Western blotting and staining with galactose specific lectin both showed that galactose was present in epimerase deficient fibroblasts and CHO cells. Gas chromatography of hydrolysed cell carbohydrates confirmed this and showed that transferase deficient cells have significantly less galactose in their glycoproteins.

Conclusions: Epimerase deficient human fibroblasts grew adequately without added galactose, and contained normal amounts of galactose produced either from residual enzyme activity or from galactoproteins in culture media. Epimerase deficient human subjects are therefore unlikely to require oral galactose supplementation. Transferase deficient fibroblasts had an abnormal pattern of glycosylation, which is likely to be related to the long-term pathogenesis of this condition in humans.

ABBREVIATIONS

BSA	Bovine serum albumin
CHO	Chinese hampster ovary
DMSO	Dimethyl sulphoxide
Epimerase	Uridine diphosphate galactose 4' epimerase
ED	Epimerase deficient cell lines
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate
Gal1P	Galactose-1-phosphate
GC	Gas Chromatography
GC-MS	Gas chromatography - mass spectrometry
Kinase	Galactokinase
NC	Normal control cell lines
PBS	Phosphate buffered saline
PHA	Phytohaemoglutanin
SDS	Sodium dodecyl sulphate
SPLL	Solid phase linked lectin
Transferase	Galactose-1-phosphate uridyl transferase
TD	Transferase deficient cell lines
VT	Versene-trypsin solution
UDPgal	Uridine diphospho galactose
UDPglc	Uridine diphospho glucose

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Chapter 1

INTRODUCTION

Preamble

In 1980 a new inherited disorder of galactose metabolism was identified at Southmead Hospital, Bristol. The discovery of this galactose intolerant, epimerase deficient patient raised questions about the effects of dietary galactose in this condition, in particular whether the deficiency was complete in which case galactose supplemetation would be required for normal growth. In this research project, cultured cell fibroblasts have been used as a model to study the requirement of these cells for galactose, and its toxic effects upon them. Lectins have been used in this work to investigate the presence and structure of complex galactosides in epimerase deficient cells. Accordingly this introduction is concerned with galactose metabolism and its disorders, with cell culture and its use in the investigation of inherited diseases, and with the use of lectins in the study of galactose containing glycoproteins.

Section 1: Galactose

1.1.1 Galactose: history and occurence

Galactose is a monosaccharide, the carbon 4 epimer of glucose. It was discovered in 1856 by Pasteur as a constituent of milk sugar or lactose, the disaccharide of glucose and galactose. Most galactose in the diet is in the form of lactose, which is the main carbohydrate source for nursing mammals and provides 40% of the energy in human milk. However galactose is more than simply an energy source for conversion into glucose, it is also an important structural component of cells, particularly the specific antigenic determinants of cell

surfaces. It is clear that although most galactose consumed is converted to glucose, the rest plays a role for which no other monosaccharide may be directly substituted.

1.1.2 Galactose as a Structural Component

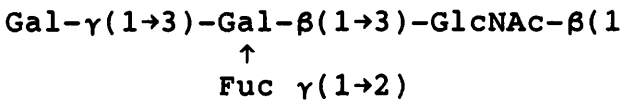
Besides existing in vivo as glycogen or blood glucose, carbohydrates are present as part of glycoproteins or glycolipids. Some glycoproteins contain oligosaccharides attached to a protein core, for example fibrinogen, the blood group substances and immunoglobulins. Others contain much longer polysaccharides combined with protein, such as the mucopolysaccharides hyaluronic acid, chondroitin sulphates, heparin keratan sulphate and dermatan sulphate. Larger carbohydrates are generally building blocks - they are present for their structural function. In contrast the function of glycoproteins that contain oligosaccharides is related to molecule or cell recognition, hence their occurance in immunoglobulins and blood groups. For instance blood group B substance is converted to H substance by removing the terminal D-galactose residues. Whether present for their structural or recognition roles it is apparent that any changes in the shape of a carbohydrate would impair or destroy the ability of that glycoprotein to function. Monosaccharides such as the epimers glucose and galactose differ only in shape rather than composition. It is clear that a correct balance of exactly the right monosacchrides is required to produce a normally functioning glycoprotein.

The number of carbohydrate groups attached to each protein ranges from one in ribonuclease B to very many in the mucins, and the proportion of carbohydrate varies from less than 1% in

collagen to 80% in blood group substances and almost 100% in the mucopolysaccharides. Galactose or N-acetyl galactosamine form a large proportion of the carbohydrates in many glycoproteins, for example it forms the majority of the carbohydrates in the repeating unit of chondroitin 4-sulphate, found in cartilage, skin and bone:

4)-β-D-GlcA-(1→3)-β-D-GalNAc 4-sulphate-(1→

and blood group B-substances:



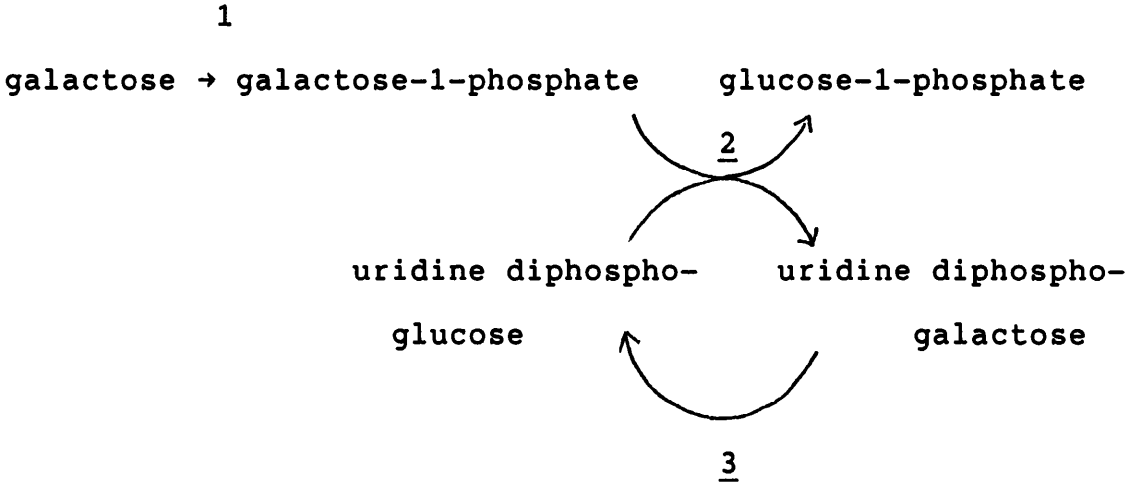
In synthesising glycoproteins the donors of the sugar units are the nucleotide diphosphate sugars. The first transylucolase is specific for the amino acid residue to which the sugar will be linked, and all transglycolases are specific for the sugar donated, and to varying degrees are specific for the acceptor sugar unit. Unlike protein synthesis there is no structural template, and control is exercised by the specificities of the transglycolases and also the levels of the nucleotide phosphate sugar donors. The sialic acids are a ubiquitous family of sugars found in glycoproteins and glycolipids and are derivatives of neuraminic acid. They appear to act as terminators of further glycosylation when attached to terminal sugars. The sugar to which they are bound is almost always galactose. In glycolipids carbohydrates play a similarly important structural role and once again galactose is a frequent component. A common example is galactosyldiacylglycerol which is a major constituent of myelin and other neural tissue.

1.1.3 Galactose Metabolism

Quantitatively the principal fate of ingested galactose is consumption as an energy source. Since it cannot be used directly the main pathways of galactose metabolism bring about its coversion to glucose. Exactly how galactose is used as an energy source was not known until the early 1950's, nearly a century after its discovery. In a number of important papers Leloir (1951) and Kalkar (1953) described the principal pathway of galactose metabolism in humans. That pathway now bears Leloir's name.

The Leloir Pathway

This allows the conversion of galactose to glucose by epimerisation of the carbon 4 hydroxyl group. Only uridine dinucleotide derivatives of these sugars are suitable substrates for epimerisation hence several enzymatic steps are required for the interchange. The pathway is shown in below:



Enzymes: 1: Galactokinase

2: Galactose-1-phosphate uridyl transferase

3: UDPglucose UDPgalactose epimerase

The Enzymes of the Leloir Pathway

The Enzymes of the Leloir Pathway

Galactokinase EC 2.7.1.6: Galactose → Galactose-1-phosphate

The first evidence for the existence of this enzymes came in 1943 with the isolation of galactose-1-phosphate from the liver of rabbits fed on high doses of galactose (Kosterlitz 1943). The enzyme has now been investigated in yeast and bacteria (Caputio 1948 and Sherman 1963) and a wide range of mammalian tissues including rat and pig liver, and human liver, erythrocytes, fibroblasts and placenta (Ng 1965, Chacko 1972, Srivastava 1972). The human erythrocyte kinase has been shown to have a dimeric structure with each component of molecular weight about 26000 (Blume and Beutler 1971). All forms of galactokinase (kinase) so far discovered need ATP as a cofactor and require magnesium ions for activity (Atkinson 1961). The reaction is reversible, but equilibrium lies far to the side of galactose-1-phosphate (gal-1-P). High concentrations of both galactose and gal-1-P have been found to inhibit the enzyme in human tissues (Mathai and Beutler 1967), a property that would tend to prevent the formation of excessive amounts of gal-1-P. In the human foetus activity can be detected as early as seven weeks gestation and increases until term (Shin-Beuring 1977). Kinase activity in the red cells of new born infants is about three times that of adults (Ng et al 1965) and there is evidence of racial polymorphism (Tedesco 1972a). In humans the kinase locus is on chromosome 17 q21 q22.

Galactose-1-phosphate uridyl transferase EC 2.7.7.12

Gal-1-P ------→ UDPgal

Discovered in 1951 by Leloir, galactose-1-phosphate uridyl transferase (transferase) catalyses the second step of the pathway. It has now been widely investigated in its structure, mechanism, kinetics and genetics. The enzyme has been found in bacteria, yeast and most mammalian tissue. The bacterial enzyme consist of two subunits, each of 40000 Daltons and the yeast enzyme is also a dimer, of 86000 total molecular weight (Saito 1967, Segawa and Fukasawa 1979). The most extensive studies have been on the human red cell enzyme but opinion differs as to its size and structure. Tedesco (1972b) using gel filtration and SDS Page proposed a trimeric structure of 90000 Daltons whereas Dale and Popjack (1976) after including uridine aminohexyl affinity chromatography produced a higher specific activity enzyme preparation with a molecular weight of 69000.

Williams (1978) proposed a dimeric enzyme, of 88000 Daltons total molecular weight by SDS Page but 67000 by gel electrophoresis. Isoelectric focusing of human liver transferase on acrylamide gel showed several bands of activity, suggesting a degree of heterogeneity (Schapira 1978). As with kinase, transferase activity is present in foetal liver, activity being highest at 25 weeks gestation. There is no change in Km during gestation, making a foetal form of the enzyme unlikely (Shin Buehring 1977).

Glucose-1-phosphate has been shown to be a potent inhibitor of transferase and uridine mono-, di- and tri-phosphates are powerful competitors of the substrate UDP-glc which itself displays inhibition at higher concentrations (Forster 1975a). From the calculated intracellular levels of these substances

it seems that the rate of reaction is regulated by substrate concentration. The inhibition by glucose-1-phosphate may explain the observation that transferase activity has been shown to be influenced by the state of carbohydrate metabolism. Perfusion of suckling rat liver with either glucose or galactose induces a rise in transferase activity initially but this falls to low levels after 90 minutes (Rogers 1981). Perhaps the fall results from an accumulation of glucose-1-phosphate, but the mechanism of the rise is not known.

The mechanism of the reaction has been investigated in bacteria and humans and found to be the same (Marcus 1977). It is of the ping pong type and is shown in the following steps, where E denotes the transferase enzyme:

E + UDPglc → UDPglc-E → UMP-E-glc1P → UMP-E + glc-1-P

UMP-E + gal-1-P → UMP-E-gal-1-P → UDPgal-E → UDPgal + E

The kinetics and mechanism of the E coli and human forms of the enzyme are the same, suggesting that selective determinants have produced a convergence of characteristics in these two phylogenetically widely separated species.

Uridine-5-Diphosphogalactose-4-Epimerase (E.C. 5.1.3.2)

UDPglc ---→ UDPgal

This enzyme and the consequences of its absence from living tissues are the main subject of this work; accordingly it will be considered in rather more detail than the other

enzymes of the Leloir pathway. Epimerase catalyses the step at which galactose and glucose are interconveted by inversion of the hydroxyl group at carbon 4. This allows the catabolism of galactose as an energy source or the synthesis from glucose of galactosides, since UDPgal is the galactosyl donor for galactoside synthesis. There is also evidence that the same enzyme is responsible for the conversion of the N-acetyl derivatives of these sugars, ie for the reaction

UDP N-acetyl glucosamine → UDP N-acetyl galactosamine.

Epimerase activity has been found in a range of organisms and tissues, including yeasts (Darrow 1966 and 1970), bacteria (Wilson 1964 and 1969), mammalian liver (Cohn 1969, Geren 1977), human fibroblasts (Chacko 1972) and human erythrocytes (Bergren 1973).

NAD has an important association with epimerase. It is a cofactor for all forms of the enzyme, whilst NADH may inhibit the reaction (Maxwell 1957). Epimerase from yeast and E. coli contains one mole of NAD for each of enzyme. The cofactor is inactive in its reduced form and is retained tenaciously by the enzyme (Maxwell 1960). Yeast epimerase is not inhibited by exogenous NADH.

Human erythrocyte epimerase

Lysates of adult human erythrocytes show epimerase activity only on addition of exogenous NAD^+, however neonatal erythrocytes do not require any added NAD (Ng 1967). Bergren (1973) showed that adult red cells contain significant amounts of NAD whereas their haemolysates do not. In neonatal erythrocytes however the levels are similar before and after lysis. It would seem that in adult's erythrocytes

intracellular NAD is destroyed during lysis by the activity of a stromal NAD nucleosidase, which is not present in neonates.

Starch gel electrophoresis of neonatal red cell lysates showed two bands of epimerase activity whereas adult erythrocyte lysates showed only one band. The addition of NAD to adult lysates produced two bands on electrophoresis identical to those of neonates, suggesting an NAD dependant structural change in the enzyme.

Cultured cell epimerase

The enzyme in cultured human fibroblasts has been characterised and cannot be induced by the addition of galactose to the cell culture medium. In cultured fibroblasts as with red cells the ratio of NAD to NADH affects epimerase activity. HeLa and L cells both show epimerase activity if NAD is added. Without the addition of NAD the cells show little activity though this can be increased by the addition of pyruvate (Robinson 1963). The enzyme optimum is pH 8.7 but at physiological pH the NADH inhibition was found to be more pronounced than at pH 8.7. Low enzyme activity in cultured cells may therefore be due to high NADH/NAD ratios and low pH resulting from anaerobic glycolysis during culture.

1.1.4 Other Pathways of Galactose Metabolism

Uridine Diphosphogalactose Pyrophosphorylase

Gal-1-P + UTP -------→ UDPgal + PP

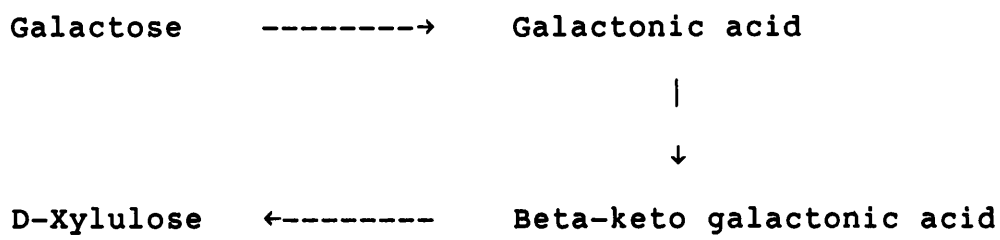
The enzyme catalysing this reaction was first described in yeast (Kalkar 1953) and then found in mammalian liver (Isslebacher 1957,1958). The human liver enzyme has been

purified and shown to be inhibited by UTP (Abraham 1969). However, when the lack of specificity of UDPglucose pyrophosphorylase became known (Lee 1978), doubt was cast upon the existence of a separate enzyme for galactose. There is now considerable evidence that the activity found is simply due to the non-specific action of UDPglucose pyrophosphorylase (Knop 1970 Chacko 1972). For instance the ratio of activity of UDPglc pyrophosphorylase to UDPgal pyrophosphorylase remains constant throughout purification, and UDPglc limits the synthesis of gal-1-P from UDPglc by haemolysates. The existence of a separate pyrophosphorylase for galactose is therefore not clear.

Reduction to Galactitol

Reduction of sugars to polyols was first described by Hers in in the seminal vesicles and placenta of sheep (1960). Galactitol has been found in the tissue and urine of humans but reduction via this pathway only occurs to any significant extent when tissue levels of galactose are very high and normal metabolism is blocked (Moonsammy 1967). Two enzymes can bring about this change: one is aldose reductase or polyol: NADP oxidoreductase, which acts on a number of aldehydes, principally glyceraldehyde (Hayman 1965,66). The other enzyme is L-hexonate dehydrogenase whose preferred substrates are uronic acid and its lactones (Mano 1961). It does act on galactose, but with much less affinity than aldose reductase. Both these pathways are only important when tissue levels of galactose are high, however this can be particularly consequential in the lens of the eye, which can be damaged by high levels of galactitol.

Oxidation via Galactonate



Dehydrogenation of the aldehyde at carbon one of galactose forms galactonic acid. This is the first step of an oxidative pathway of galactose metabolism described by Cuatrecacas and Segal (1966). They characterised the enzyme galactose dehydrogenase and proposed it as the first step of the pathway shown above, to form D-xylulose, a sugar capable of further metabolism. However, Srivista (1969) failed to demonstrate the oxidation of galactose to galactonate but did give evidence for the formation of galactose-6-phosphate and its subsequent oxidation to 6-phosphogalactonic acid. Galactonate is excreted in the urine of humans whose normal pathways of galactose metabolism are blocked (Bergren 1972).

Section 2: Disorders of Galactose Metabolism

1.2.1 History

The first detailed report of a disease involving galactose metabolism was by Goppert (1917). He described a two year old child who had an enlarged liver, delayed mental and physical development, and galactose and albumin in his urine. This child had severe icterus until 8 months of age. Goppert found that putting the child on a milk free diet caused him to improve markedly: the liver reduced in size, sugar and albumin disappeared from the child's urine and his mental and physical development improved.

The term galactosaemia is now used to describe the inherited disoders caused by the absence of an enzyme required for galactose metabolism. Lesions have been found at the level of transferase, kinase and epimerase. These all result in elevated levels of galactose in the blood, ie galactosaemia. The term should therefore be qualified by mention of the missing enzyme. Goppert's case was most probably transferase deficiency galactosaemia.

1.2.2 Galactokinase deficiency

This defect was first described in a blind 44 year old Swiss gypsy by Gitzelman in 1965. The subject had recurrent cataracts and mellituria as a child. He was investigated by Fanconi at that time who identified the sugar in the childs urine as galactose and termed the condition galactose diabetes. Thirty-five years later Gitzelmann showed that the same subject lacked erythrocyte galactokinase and that his conversion of radiolabelled galactose to carbon dioxide in

vivo was minimal, suggesting that the enzyme was missing from all tissues.

The only clinical symptom of the deficiency is the occasional occurrence of cataracts. Deficient subjects show no aversion to galactose or lactose and it is noticeable that the kidney and liver damage and mental retardation of transferase deficiency do not occur. Treatment is by the exclusion of galactose from the diet. This is advised in order to inhibit the formation of cataracts. Studies in Gitzelmann's subjects showed that ingested galactose was excreted as galactose and galactitol in the molar ratio of about 4:1 (1974). Only about 5% of labelled galactose activity appeared in expired air, an amount similar to that produced by transferase deficient galactosaemics, probably by the decarboxylation of galactonic acid. Since epimerase and pyrophosphorylase are functioning kinase deficient subjects should be able to synthesise sufficient galactose for developmental requirements from glucose while they are on a galactose free diet.

The disorder is transmitted by autosomal recessive inheritance. Population studies have shown a heterozygote frequency of about 1% which would give a birth incidence of 1 in 40000 (Gitzelmann 1967). However, the observed incidence in Germany and Austria was found to be only about 1 in 155000 (Gitzelmann 1980). No doubt this discrepancy is in part due to some cases being missed, but genetic heterogeneity may contribute: the first four cases found were unrelated gypsies so it is possible that this group has a higher incidence of carriage of the gene for kinase deficiency.

1.2.3 Hereditary transferase deficiency

This is the most common disorder of galactose metabolism and the one which has been the most extensively investigated. Although galactosaemia was recognised as a disorder from the early part of the century, the location of the enzyme defect was not discovered until much later. In 1956 Schwarz found elevated levels of gal-1-P in the erythrocytes of patients with galactosaemia. This led Isslebacher (1956) to assay the enzymes of the Leloir pathway and show that red blood cells from galactosaemics specifically lacked transferase. The deficiency has now been shown to exist in cultured fibroblasts, amniotic fluid cells and in leucocytes and liver tissue. Transferase deficiency is transmitted as an autosomal recessive trait and heterozygotes for the galactosaemia gene have about half normal activity.

Affected children usually present in the neonatal period. They have normal birth weights but gain little after they start milk feeds. Symptoms usually appear in the second half of the first week of life, and include jaundice, vomiting, lethargy, aminoaciduria, galactosuria and cataracts. If a galactose-free diet is instituted symptoms resolve promptly. However some degree of irreversible brain damage may occur. The average IQ of a group of British galactosaemics was 80, and was found to decrease with age. However galactosaemics in whom intrauterine exposure to galactose was kept to a minimum by restriction of the maternal diet were found to have virtually normal distribution of IQ's (Fischler 1980). Treatment with a galactose free diet can be monitored by measurment of erythrocyte gal-1-P.

1.2.4 Galactose metabolism in transferase deficiency

Initial investigations were carried out in vivo in galactosaemic subjects to establish whether they could metabolise galactose at all, and if so by which pathways. In a group of 14 galactosaemics, nine showed between 0 and 8% conversion of labelled galactose to CO_2 and five, all black had near normal rates (Segal 1968). The poor converters were all Caucasians and did not improve their ability to metabolise galactose when monitored over several years. Biopsies of liver and intestinal mucosa from black subjects showed about 10% of normal activity. It was concluded that black subjects have some capacity to oxidise galactose because of residual activity in their visceral tissues. That they showed clinical features of galactose toxicity in the neonatal period may be due to the large amount of galactose ingested then. Accumulation of gal-1-P in galactosaemics is probably not exclusively due to the phosphorylation of galactose by kinase. In both normal and transferase deficient subjects it may be formed by the action of epimerase and pyrophosphorylase:

$$\text{UDPglc} \xrightarrow{\text{epimerase}} \text{UDPgal}$$

$$\text{UDPgal} + \text{PP} \xrightarrow{\text{pyrophosphorylase}} \text{gal-1-P} + \text{UTP}$$

There is considerable evidence that gal-1-P is formed in galactosaemics by this pathway. Tranferase deficient fibroblasts accumulate gal-1-P in a galactose free medium

(Pourci 1990). One newborn galactosaemic was given parenteral nutrition only for his first 24hr; however his erythrocyte gal-1-P rose over this period (Gitzelman 1975). Transferase deficient newborns have raised gal-1-P even though their mothers abstain from galactose during pregnancy. However experiments with labelled galactose show a different yield of CO_2 when carbon-1 or carbon-2 labelled galactose is given: this is consistent with direct oxidation to galactonate followed by decarboxylation. No difference would be found if oxidation occured via pyrophosphorylase. Therefore it seems that the pyrophosphorylase pathway can contribute to the formation of gal-1-P but not its breakdown which is surprising. The true importance of this pathway in galactosaemia has probably yet to be established.
One pathway that is clearly functioning is reduction of galactose to galactitol by aldose reductase, an NADPH requiring enzyme with a broad specificity for aldehydes. Galactitol has been found in the tissue and urine of galactosaemics and was shown to be formed from labelled galactose (Quan-ma 1966). Galactitol is not further metabolised, the main route of elimination being excretion in the urine. This continues for several days after galactosuria has stopped.

1.2.5 Incidence of transferase deficiency

Transferase deficiency galactosaemia is transmitted as an autosomal recessive trait. Heterozygotes for the galactosaemia gene have about half normal activity. Several studies have investigated the incidence of transferase deficiency in a

number of areas, either from the results of neonatal screening or the detection of heterozygotes. Six million newborns have been screened worldwide with a detection rate of 1 in 62000 (Levy 1980). Estimates based on the detection of heterozygotes in Wales, Denmark and the United States gave figures ranging from 1 in 18000 to 1 in 180000. The observed prevelance at birth was 1 in 70000 (Schwarz 1961). Various factors could account for this range of figures. These could include heterogenious distibution of the enzyme between various populations, differences in the effectiveness of screening programmes and errors resulting from small sample size. Prevalence at birth will give an underestimate in the likely event of some cases being missed.

1.2.6 Transferase polymorphism

In 1966 Beutler described the Duarte variant of transferase which has reduced activity in red cells and other tissues. Heterozygotes have about 75% of normal activity and homozygotes 50%. The Duarte variant is indistinguishable by Michaelis constant, pH optimum or thermal stability but it migrates faster on starch gel electrophoresis (Beutler 1966). The most common cause of an abnormal result in galactosaemia screening is double heterozygosity for galactoaemia and the Duarte variant. Schwarz (1982) found that challenging these subjects with a galactose load resulted in raised levels of blood galactose and gal-1-P. They suggest that such a marked abnormality in the ability of these subject to metabolise galactose raises questions about the appropriate dietary recomendations for them.

A number of other variants have now been described and all except the Los Angeles variant have lower activity than the wild type. In combination with the gene for classical galactosaemia most variants can produce moderate symptoms such as raised blood galactose and galactosuria. The Indiana variant was described in an 18 month old Caucasian girl who had galactosuria and raised red cell gal-1-P and blood galactose when challenged with milk (Chako 1971). Her erythrocyte activity was about 35% of normal, but was highly unstable and could not be detected on electrophoresis. An elder sib of this case had died at six weeks with symptoms of galactosaemia.

1.2.7 Epimerase Deficiency

Epimerase is the only known enzyme which actually interconverts glucose and galactose in any form. A deficiency was thought to be incompatable with life (Kalkar 1965). The absence of epimerase in any organism would produce a galactose auxotroph, ie one that would require dietary galactose for the synthesis of galactosides from UDPgal. It would also be unable to dispose of ingested galactose by converting it to glucose.

However Gitzelman reported nine individuals whom he described as being epimerase deficient (1980). Five were discovered on neonatal screening for galactosaemia and the other four were relatives of the affected neonates. All subjects were asymptomatic and needed no dietary restriction of galactose. The only abnormality in these cases was raised erythrocyte gal-1-P which was responsible for the positive screening

tests. The condition seemed to be benign because the defect was restricted to circulating erythrocytes and leucocytes.

In all cases where epimerase activity was measured in liver or fibroblasts at least 50% of normal activity was found. The condition was transmitted as an autosomal recessive trait. The incidence is hard to estimate because of the lack of symptoms, but the Swiss study showed an incidence of 1 in 46000.

Then Holton et al (1981) reported a severly ill baby girl with symptoms similar to transferase deficiency galactosaemia but with normal transferase activity. This child was found to be deficient in erythrocyte epimerase, and subsequently also in cultured fibroblast epimerase (Gillett 1983). Further work on this index case by Gillett (1985) suggested that this subject did possess some epimerase activity: about 11% of control levels were found in liver tissue in a biopsy from her. When she was put on a galactose-free diet her jaundice abated, her liver function tests returned to normal and she thrived. When challenged with galactose her symptoms returned. The severity of her symptoms would suggest a different disorder from that described by Gitzelman, involving a more widespread deficiency.

Some galactose was included in her diet to allow the synthesis of galactosides but it has been difficult to find the correct level of supplementation (Henderson et al 1983). The ingestion of even small amounts of galactose result in a rapid rise in her red cell UDPgal but the concentration of gal-1-P was proportional to her galactose intake. The patient

did not sit until 15 months old and had a development quotient of 70 at 19 months.

A second case of generalised epimerase deficiency was reported by Garibaldi et al (1983). They claimed that no epimerase activity could be detected in erythrocytes or fibroblasts, and that parental fibroblasts showed reduced epimerase activity. These findings suggested a similar defect to that described by Holton et al.

A third case has been reported (Sardharwalla 1988). this was a child whose aminoaciduria was detected by a newborn screening programme. She was unwell with poor feeding, vomiting, jaundice and hepatomegaly, and had to be treated for a staphylococcal septicaemia. She was given a galactose-restricted diet (not galactose-free) containing 30ml of cow's milk or 1g of galactose per day. At 2 years and 9 months she was severely mentally retarded with an IQ of 55 and had, importantly, profound sensori-neural deafness as did the index case reported by Holton. The amount of galactose required by children such as these is not known, and neither is the consequence of over or under supplementation. One of the purposes of the work described in this thesis is to enable reasoned choice of the amount of galactose, if any, required by such infants and to avoid the arbitary choice of dietary galactose content without knowledge of its requirement or consequence.

The only other report of an organism which had a consequential lack of epimerase was from Nikaido et al on mutants of salmonellae (Nikaido 1961, Fukasawa 1961). They found these cells to be deficient in epimerase and to accumulate intracellularly large amounts of UDPgal and gal-1-P when grown

in medium containing galactose. Nikaido analysed the sugars in the cell walls of these mutants. Surprisingly the only sugar present was glucose, and they lacked any galactose, mannose, rhamnose or tyvelose which were present in the wild type cell wall.

1.2.8 Galactose toxicity

In transferase deficiency galactosaemia, exposure to galactose causes damage to brain, liver, kidney and the lens of the eye. Attempts to investigate galactose toxicity have been hampered by the lack of a suitable animal model. Diets high in galactose have been tried in chicks and young rats and can induce raised blood galactose, but since all enzymes are present the pattern of accumulation will not be the same as in the human galactosaemic. The toxic effects of galactosaemia vary from organ to organ, accordingly they will be considered individually.

The lens: this is damaged in transferase and kinase deficiency, indeed it is the only tissue known to be damaged in kinase deficiency. The formation of cataracts is undoubtedly associated in galactosaemics with the presence of galactitol. Aldose reductase is present in large quantities in the lens, and an inhibitor of that enzyme delayed cataract formation in galactose-fed rats (Peterson 1979). Galactitol is formed within the lens and diffuses out only with difficulty. The osmotic activity of galactitol in the lens is a likely cause of cataracts since in vitro incubation of lens in a medium whose osmolality is increased to balance the galactitol formed stops cataract formation.

The liver: this is damaged in transferase deficiency but the pathogenesis is not well understood. There are however indications that a deficiency of nucleotides is a cause. Keppler et all have demonstrated increased catabolism of hepatic nucleotides in galactosaemics after a galactose load (Forster 1975). More recently they have shown that D-galactosone, a galactose analogue metabolised in the same wayas galactose causes a deficiency of UTP in hepatoma cells (Keppler 1982).

The kidney: This is another tissue damaged in transferase deficieny. Gal-1-P and galactitol have both been found in the kidneys of transferase deficient subjects. However the aminoaciduria of transferase deficiency is not seen in kinase deficient subjects, who excrete large amounts of galactitol. Amnioaciduria has been induced in normal subjects to whom galactose was given intravenously (Fox 1964). Incubation of rat kidney cortex slices produces non-competitive inhibition of aminoacid accumulation by tubule cells. Thus it seems that gal-1-P rather than galactitol is the toxic agent and that it induces its effect by disturbing aminoacid transport.

The Brain: The manifestation of galactose toxicity which is argueably the least understood and most serious is mental retardation. Galactitol has been found in brains of subjects with transferase deficiency, and in rats fed galactose the level of accumulation in the brain is second only to that in the lens. However, the lack of any retardation associated with kinase deficiency makes a pathological role for galactitol unlikely. It has been hypothesised that a decrease in serotonin receptors is the cause, but no abnormality has

been observed in the uptake of serotonin by the synaptosomes of galactose intoxicated rats (Yandrasitz 1979). However, interpretation of work on rats is difficult since they do not show acute brain toxicity on high galactose diets. The only fact that can be stated with certainty in this field is that the mechanism by which galactose causes toxicity in the brain remains to be found.

The immune system: Disruption of the immune system is not normally quoted as being on of the effects of galactosaemia but it is becoming apparent that it is a very significant and highly consequential effect of the disorder. Among 35 infants with classical galactosaemia discovered on newborn screening ten had systemic infection of which nine died despite therapy (Oski 1979). Escherichia coli was the causative organism in most cases. These observations prompted Kobayashi et al (1983) to investigate in vitro neutrophil function using neutrophils from normal adults and neonates. They found that neutrophil function was depressed by galactose in adults and to an even greater extent in children. It is therefore clear that galactosaemia can compromise the immune system, and that this can have fatal consequences so rapidly that neonatal screening does not prevent death.

The ovary: There have been reports of a very high incidence of gonadal failure in female galactosaemics (eg Kaufman 1981, 1988, 1989; Dessart 1982). Twelve out of 18 female galactosaemics had hypergonadotrophic hypogonadism and 11 had amenorrhoea. One suffered ovarian failure after an uneventful pregnancy and the birth of a normal child. Gonadal function in 8 male galactosaemics was normal. The mechanism by which

this occurs is not known, but the possibility of toxic destruction of the ovaries during intrauterine life by a galactose metabolite has been suggested (Dessart 1982).

Cellular toxicity: galactose toxicity is not limited to whole organs,toxic effects have been demonstrated against cells in culture. The growth of fibroblasts from transferase deficient subjects is inhibited by adding galactose to the medium. When these cells are incubated with galactose, electron microscopy shows dilation of the endoplasmic reticulum. Cytoplasmic degeneration and cell death occur within 3 days (Miller 1968). Galactosaemic fibroblasts also have impaired metabolism of sulphate when exposed to galactose (Tedesco 1979).

1.2.9 Toxicity in epimerase deficiency

The wide difference in clinical consequence between lesions of different enzymes in the same pathway is well illustrated by the contrast between kinase and transferase deficiency. However, it may be possible to hypothesise about some effects of epimerase deficiency in the light of our knowlege of the other defects of galactose metabolism. In epimerase deficiency gal-1-P is raised to a similar level to that found in transferase deficiency (Henderson et al 1983). Since gal-1-P has been implicated as causing the majority of the toxic effects of transferase deficiency then it is likely that these will be a feature of epimerase deficiency. Indeed abnormally high liver function tests, jaundice and aminoaciduria were observed in the reported case. This child also had markedly elevated UDPgal when not on a galactose-free diet. UDPgal is the substrate for galactosyl transferases

which are responsible for galactosylating glycoproteins and glycolipids. It is possible that a galactose containing diet could induce greater degrees of galactosylation in such a subject by increasing the amount of substrate, and conversely a galactose-free diet could result in lower levels of galactosylation. The consequences of this are not known.

Section Three: Cultured Cells and Inherited Disease

1.3.1 The history of cell culture

In 1907 Ross Harrison grew frog nervous tissue in clots of frog lymph, and it is generally accepted that this was the first true tissue culture. Techniques for culturing tumour cells developed slowly from that time. In 1914 Losee Ebelling was culturing human tumour cells and by 1936 Gey was able to do this on a considerable scale. He also showed that the culture of normal human cells was not as difficult as previously thought. However transformed lines like Hep 1 and HeLa which appeared to grow indefinitely were easier to use, and the degree to which these lines were abnormal was not fully realised at the time.

When the normal chromosome number was found to be 46 in 1956 the use of primary cells received considerable stimulus, since these perpetual cell lines were found to be chromosomally abnormal.

Fortunately techniques soon developed for culturing normal fibroblasts. Puck (1958) showed that it is possible to grow cells from small skin biopsies by trypsinising the tissue and seeding out the cells. The plasma clot explant technique which was developed for growing fibroblasts from skin biopsies has also been used for many other tissues. At first human skin fibroblast cultures were used mainly for cytogenetic studies, then in 1960 Krooth and Weinberg found that fibroblasts from patients with galactosaemia failed to grow in a medium with galactose as the only energy source. Since then cultured cells have been used in many studies relating to inherited disease.

1.3.2 How cells are cultured

It is possible to grow cells from samples of almost any tissue. Some like liver are particularly difficult to grow, and from others a mixed population of epithelial cells and fibroblasts will grow, but fibroblasts always come to predominate. Almost pure epithelial cells can be obtained from tissues such as the kidney. However the vast majority of culture is carried out on samples that can be collected routinely from any subject without major risk, that is skin, blood and amniotic fluid.
A skin biopsy may be obtained easily and almost painlessly by picking a fold of skin with forceps and cutting of the top with a scalpel blade.
The biopsy is transported to a laboratory in culture medium. On arrival it is cut up into small pieces and these are anchored in culture vessels, for instance with clots of serum. Cells will grow out from the pieces of skin and after several days sufficient should be present for subculturing by removing them with a mixture of trypsin and EDTA.
Primary human cells cannot be cultured indefinitely but die out after about 50 - 60 doublings for embryonic fibroblasts and 30 - 40 for those of adults (Hayflick 1965). Cells with metabolic abnormalities may not even grow for this period.

Amnotic fluid cells

Cells from amniotic fluid are harder to grow than those from skin biopsies. Normally a few mls of amniotic fluid are mixed with cell culture medium and incubated in a culture vessel. Once established the cultures can be handled in a similar manner to fibroblasts.

Blood culture

This may be either long or short term. For short term culture whole blood or purified lymphocytes is mixed with culture medium containing phytohaemagluttinin (PHA) or another similar mitogen. After 48-72 h large numbers of cells are in mitosis. However the cells will only continue to divide for two or three cycles which limits the usefulness of this method. Long-term culture of lymphoid cell lines may be established by exposing the cultures to high concentrations of Ebstein-Barr virus (EBV). Virus transformed B lymphocytes will then grow out. If such cells are used for the study of inherited disease then it should be remembered that the EBV genome is expressed in these cells. One advantage with B lymphocytes is that since the cells grow in suspension it is possible to grow very large numbers quite easily.

1.3.3 Cryopreservation

Cultured cells may be stored for long periods in liquid nitrogen and yet remain viable, ie capable of growth and division on thawing.

Cells must be frozen in the presence of a cryoprotective agent such as DMSO. The rate of cooling is important, normally cells are cooled slowly (about 1°C per minute) to -30°, then quickly (10° per minute) to -196°. Cells may be stored for many years in this state.

1.3.4 Cultured cells and the diagnosis of inherited disease

Many inherited diseaes may be diagnosed by establishing the existence of an enzyme deficiency in cultured cells, indeed I-cell disease takes its name from inclusions found in cultured cells. This is a relatively recent advance which has become possible as the exact nature of the lesion in many inherited diseases has been found.

Substrate accumulation in vivo is often a useful indicator of the cause of a disorder, but when a diagnosis remains with an affected case for life then the exact nature of the enzyme defect should be established if possible. Erythrocytes are the most easily obtained cell but they contain only cytoplasmic enzymes, and lacking nuclei they cannot be cultured. They can however be used for the study of cytoplasmic enzyme abnormalities such as hypoxanthine guanine phosphoribosyl transferase deficiency in Lesch-Nyhan syndrome (Dancis 1973). Leucocytes and fibroblasts may be used as relatively undifferentiated cells, ie ones in which a large number of enzymes are expressed which are not relevant to the particular function of that cell. For most diseases both polymorphonuclear leucocytes and cultured skin fibroblasts may be used for diagnosis (Milunsky 1972). However if cells have to be stored before or after enzyme assay then fibroblasts are normally used since transformation is not necessary.

1.3.5 Cultured cells as models for inherited diseases

Cultured cells from a subject with an inherited disease may be used as a model for that disease in preference to carrying out experiments on that subject. Indeed the first use of cultured

cells in the study of inborn errors was as a model rather than for diagnosis when Krooth and Weinberg showed that fibrolasts from a patient with galactosaemia failed to grow in a medium with galactose as its main energy source (1960).

Since then many inherited diseases have been investigated in culture ranging from sulphatase deficiency (Roy 1977) to gangliosidoses (Galjaard 1977) and familial hypercholesterolaemia (Robertson 1977).

Fibroblasts are normally preferred since cells are usually cultured for a longer time than for diagnosis. Transformation would therefore be required for lymphocytes, but for fibroblasts no viral or mitogenic stimulation is needed, adding to their validity as a model.

If it is merely the intracellular biochemistry that is under examination the fibroblasts provide an excellent model since very large number of enzymes are expressed in them. The external biochemical environment can also be controlled for cultured cells in a manner not possible in vivo, and radiolabelled compounds can be fed to cells and their catabolism followed, which also may not be possible with living subjects.

There are however limitations to the use of cultured cells as a model for inherited disease. Some enzymes are simply not expressed in fibroblasts, often those which are found exclusively in liver such as phenylalanine hydroxylase, whose deficiency causes phenylketonuria.

Even if the enzyme under consideration is expressed then there is a possibility that other relevant enzymes may not be present, such as those synthesising toxic by-products from

metabolites accumulating behind an enzyme block, thus giving a misleading picture. Lastly such a model cannot give information about a damage to a particular tissue, for instance the lens of the eye or ovary in galactosaemia.

1.3.6 Problems with cell culture

There are several other problems that arise in the practice of tissue culture. One is that the metabolism of cells in culture may not be the same as that in vivo. Rapidly proliferating cells are often said to de-differentiate (Hardnen 1977). They acquire high levels of glycolysis and there is evidence for switches in enzyme function.

For instance Owens and Nebert (1975) showed that in a wide range of cultured tissues the cytochrome P450 oxidase is hard to activate, whereas the P448 which is characteristic of more rapidly dividing cells seems to take over its function. Drugs which induce P450 in vivo induce P448 in culture.

A second problem is contamination of the culture. Bacterial contamination could conceivably lead to erroneous results but is usually obvious. Infection with mycoplasma is usually more occult and is known to cause alterations in metabolism and chromosomal abnormalities. It has also been shown that mycoplasma infection can produce apparently normal enzyme activities in cells deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRT) (Stanbridge 1975) and induce a 10-fold increase in pyruvate dehydrogenase activity in deficient cells. Mycoplasma may be detected in cultured cells by staining with a fluorescent dye and examination under a UV microscope.

Continuous vigilance of cultures is obviously important. Contamination with virus is easily detected since it usually destroys the culture, however it is a rare occurence. The last source of contamination is other cells. It has been shown that a large proportion of established cell lines are in fact HeLa cells (Gartler 1968). Fortunately cells from patients with inborn errors have built-in markers.

One final problem is the artifical correction of a defect by absorptive pinocytosis of the missing enzyme from the cell culture medium by deficient cells. This has been documented in mucopolysaccharidoses (Neufeld 1973). Media conditioned by normal cells have enough enzyme activity to normalise mucopolysaccharide storage in mutant cells. It has since been shown that normal cells can transfer to mutant cells N-acetyl β-D-hexosaminidase, but not a glucosidase or β-galactosidase. It has been recognised that human serum should not be used for culture in this field because of the possible presence of corrective factors.

1.3.7 Culture medium and fibroblast metabolism

The environment of cultured cells consists of the culture medium, a solid support on which cells grow and a neighbouring gas phase.

The principal influence on the metabolism of cultured cells is the composition of the medium. Initially media were derived from body fluids, now synthetic media are used to which serum is added as a supplement. The basic components of a synthetic medium are a salt mixture, an energy source and some vitamins. In addition a buffer is needed to maintain the pH and a serum

content of between 2 and 20% is necessary for diploid cell growth and division. All these components may influence the metabolism of cells.

Salt mixture

Medium must be of an appropriate osmotic tension, about 300 mosmol/kg, the bulk of which is contributed by sodium chloride, but fibroblasts can tolerate quite large changes (about 10%) in osmotic pressure without apparent effects on metabolism (Paul 1975). Other ions are essential components of cell metabolism. Magnesium and calcium are required to anchor cells to surfaces and are important in many stages of intermediary metabolism. Magnesium for instance is necessary in all transphosphorylation reactions. Zinc and iron are required for cell growth but the need for other trace elements is unclear. The only recorded effect of salt composition on cells with inborn errors of metabolism is the ability of magnesium concentration to affect HGPRT (Benke 1973), however other enzymes may show dependance on divalent cation concentration.

Energy source

The most widely used energy source is glucose though other saccharides may replace it. The metabolism of glucose is mainly glycolytic, resulting in the accumulation of lactate in the medium. However, cells do not possess all the enzymes necessary for respiration and the pentose phosphate shunt, and the balance between glycolysis and respiration can be varied according to cultural conditions.

Inhibition of respiration by anaerobic conditions causes increased glycolysis (the Pasteur effect) while increased

concentrations of glucose may inhibit respiration (Crabtree effect). Increased glucose concentration may also promote glycolysis.

The first metabolic deficiency detected in fibroblasts was galactosaemia, shown by the inability of the fibroblasts to use galactose instead of glucose as an energy source. It has also been shown (Zacchello 1972) that galactose in the medium will induce galactokinase activity in normal subjects and heterozygotes for kinase deficiency.

Amino acids: No cultured cells have been shown not to grow in the absence of eight essential amino acids required by man. In addition five other amino acids are required in vitro: glutamine, arginine, tyrosine and histidine which are synthesised in the liver of intact organisms, and cystine. Non-essential amino acids are often added to stimulate growth. No studies have shown any consequences of amino acid composition on metabolic processes relevant to inherited diseases.

Vitamins: Eight vitamins are required by cultured cells, six of which function as co-enzymes. There are specific inherited diseases involving vitamins, but they can also affect cells with other abnormalities. Vitamin C causes a marked rise in intracellular mucopolysaccharides in cells from patients with Hurler's syndrome (Schafer 1966) and a less marked change in normal cells. The B vitamin responsive errors of metabolism are well known and this response can be shown in tissue culture. For instance the addition of 5'deoxyadenosyl-cobalamin corrected the metabolic abnormality in cells from a patient affected with methylmalonic acidaemia (Kaye 1974).

pH and carbon dioxide: Cells in culture must be maintained within narrow pH limits in order to grow properly. Until recently the usual buffering system was bicarbonate / carbon dioxide which gave an appropriate pH when in equilibrium with 5% CO_2 in the gas phase. However this requires an artificial gas phase and is not a very strong buffer since the required pH is some way from the pKa of bicarbonate at 6.1. Organic buffers such as Hepes are now often used since they are more effective buffers and do not significantly change pH with temperature. However it does seem that bicarbonate is essential for normal cell growth, possibly because the absence of CO_2 causes decarboxylation of oxaloacetate with consequent cessation of cell respiration and there is also a CO_2 fixing step in de novo ATP biosynthesis. Oxaloacetate restores growth in CO_2 deprived cultures. Changes of pH have many effects on metabolism including on morphology, glycolysis, growth, membrane transport, mucopolysaccharide production and collagen production (Lie 1977).

Serum: Completely synthetic media can be used for some heteroploid cell lines but human diploid fibroblasts require serum for growth. Serum has many effects on metabolism, increasing transport of various nutrients and stimulating protein and DNA synthesis. The main effect detected which concerns inherited diseases is the alteration in lysosomal enzymes with changes in serum concentration, for instance B-galactosidase tends to be lower at higher serum concentrations (Lie 1977).

1.3.8 Galactosaemia and cultured cells

As already mentioned, the paper by Krooth and Weinberg was the first on inherited disease cells in culture. In it they showed that while normal fibroblasts grew equally well with 100mg% galactose or glucose as their energy source, transferase deficient cells grew normally with glucose but equally slowly in a galactose or a hexose-free medium. Heterozygotes gave variable results but were predominantly equal in galactose or glucose. They also compared growth in media containing 95mg% galactose and 5mg% glucose with that containing just 5 mg% glucose. Normal and heterozygote cells grew equally well in the two media but transferase deficient cells grew better in just 5mg% glucose, and growth was slowed for the first 72 hours by adding galactose. Deficient cells were not able to produce $^{14}CO_2$ from radiolabelled galactose, unlike normal controls.

Miller and coworkers examined the cytologic effect of media containing galactose on galactosaemic fibroblasts in culture. They observed changes they describe as remarkable using 100 mg of galactose per 100 ml of media (Miller 1968). Loss of electron dense material in the lumen of the rough endoplasmic reticulum was followed by massive dilatation of the reticulum tubules. Degeneration and cell death were evident after 72 hours. The galactose containing medium was selectively toxic for the galactosaemic line.

Work done on a cultured cell model for familial hypercholesterolaemia has recently been found to have a fascinating relevance to galactosaemia. Krieger et al (1983) produced a CHO mutant cell line defective in low-density

lipoprotein (LDL) receptors by growing cells in a high concentration of amphotericin B. These cells have subsequently been found to have no measurable epimerase activity and it has been proposed that their inability to synthesis galactose prevents the formation of LDL receptors, of which galactose is an essential component. They therefore would seem to be a cellular model for complete epimerase deficiency.

Other relevant work has been carried out in cell lines which do not have disorders of galactose metabolism. King and Pope (1984) showed that all-trans retinoic acid increased the rate of incorporation of galactose into glycoproteins in cultured pig skin slices. The incorporation of glucosamine and leucine were not affected, and there was no evidence for the synthesis of new types of glycoprotein. Schwarz and coworkers examined the ability of brain cells from neonatal mice to metabolise galactose (1985). They found that galactose was much less readily consumed than glucose and that even low levels of glucose could reduce the uptake of galactose, whereas galactose at concentrations of up to 11.6 mmol/l did not inhibit glucose uptake.

Lactate was consumed from the medium at all galactose concentrations whereas glucose caused the release of lactate by cells. They concluded that galactose is poorly metabolised by brain cells but does not exhibit toxic effects.

The addition of galactose to culture medium has been shown to protect CHO cells from a thermal shock to 45°C to a greater extent than either glucose or mannose (Henle 1984). Neutra and Leblond (1966) showed that when labelled galactose was taken up by cells it was located first in the Golgi region then in

secretory products such as mucins. They concluded that the synthesis of complex carbohydrates takes place in the Golgi region of many secretory cells. The other area of study combining galactose and tissue culture relates to malignancy. Klohs et al (1984) found that UDPgal inhibited the growth of mouse leukaemic L1210 cells by up to 92%. Other nucleotide sugars as well as galactose, glucose and gal-1-P had no effect. This inhibition was not observed with lines such as 3T12, which did not secrete galactosyl transferase activity into the medium. Normal growth was resumed when UDPgal was removed from the medium. They suggest that UDPgal inhibition of cell growth requires galactosyl transferase activity, and that the effect is mediated via the cell membrane. They hypothesise that either the addition of galactose to a cell surface glycoconjugate signals the cell to stop dividing, or that it prevents hormones or growth factors from binding to the membrane. The implication for these findings on disorders of galactose metabolism have not been considered. Other work has investigated the production of abberant cell surface carbohydrates by malignant cells, for example 56% of labelled galactose incorproated into F9 embryonal carcinoma cells was found on the plasma membrane (Kawamoto 1983).

Section 4: Lectins

1.4.1 Introduction

A lectin is a carbohydrate binding protein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates (Goldstein 1986). Most lectins come from plants; however they have also been found in a range of organisms including bacteria, sponges, fish, molluscs and mammals. No purified lectin has been shown to exhibit enzyme activity. Lectins were discovered during work to isolate the highly toxic principle of various seeds, including castor beans and jequirity and croton seeds. Extracts of the cells were found to agglutinate red blood cells, and research was stimulated by the realisation that certain lectins could be used as blood typing reagents. Since then, they have found many other uses as experimental tools. As mitogenic stimulants they have wide application in immunology and cytogenetics. As structural probes lectins have revealed much about the organisation of the cell surface, and its changes during the cell cycle and malignant transformation. Lastly, the saccharide binding properties of lectins have been used in a number of analytical and preparative procedures for characterising, sequencing and purifying carbohydrates, glycoproteins and glycopeptides. All of these effects of lectins are inhibited or abolished by the carbohydrate for which the lectin is specific.

1.4.2 Specificity

In order to interpretate the results of experiments with lectins their carbohydrate binding specificity must be known.

Sugar - lectin complementarity is usually determined by the Landsteiner hapten inhibition technique (Landsteiner 1962). This is done by comparing sugars on the basis of the minimum concentration required to inhibit the precipitation reaction between a lectin and a reactive macromolecule, or the haemagglutination reaction. A number of other procedures have given information on carbohydrate binding specificity, including elution from solid phase adsorbents, precipitation studies with synthetic carbohydrates (eg Lonngren 1976); ultraviolet spectroscopy, fluorescence spectroscopy and equilibrium dialysis.

The C-4 hydoxyl group of carbohydrates is critically involved in lectin binding. Galactose binding lectins do not interact with glucose or mannose (Hayes 1974, Nicholson 1974). This makes them particularly useful for detecting galactose in the presence of other carbohydrates.

Some lectins have been shown to contain sites which bind non-carbohydrate compounds. Concanavalin A and a number of leguminous lectins bind fluorescent hydrophobic molecules such as 1,8-anilino naphthalenesulphonic acid. Lima bean lectin has a single high affinity site for adenine, as do lectins from Dolichos biflorus, Phaesolus vulgaris and Glycine max (Roberts 1983).

1.4.3 Ricin Lectin

Lectins from the castor bean, Ricinus communis has been used in several of the experiments described in this thesis. Aqueous extracts of the castor bean contain two distinct but structurally related lectins: a very cytotoxic weak

haemagglutinin and a strong erythroagglutinin that is only a weak inhibitor of protein synthesis (Olsnes 1978). These two components are termed ricinus communis toxin (ricin or RCA II) and ricinus communis agglutinin (RCA I). They can be separated by a number of affinity chromatography methods, for example sequential elution from Sepharose 4B of the toxin with N-acetyl hexosamine and the agglutinin with galactose.
RCA I is a tetramer of molecular weight 120000 Daltons. It has a large number of interchain disulphide bonds, and perhaps because of this it is a robust molecule, resistant to repeated freezing and thawing, pH changes and proteolysis (Olsnes 1976, Ishigino 1964, Olsnes 1975).
RCA I binds galactose and galactosides, with the specificity:

beta-galactose > alpha-galactose > N-acetylgalactose

(Goldstein 1986). It binds two molecules of the saccharide with Ka = 1.24×10^3 and 3.8×10^4 per mole for galactose and lactose respectively, at 4°C (Goldstein 1986). The relative inhibitory potencies of a number of sugars are shown in table 1 from Goldstein 1986. From this table it can be seen that lactose is 7-8 times more potent an inhibitor of agglutination than galactose. This, combined with the other results in the table suggest that the binding site of the agglutinin is an extended one, capable of binding to the glucose unit of lactose and probably interacting with the C-4 and C-6 hydroxyl groups of the beta galactosyl unit. Extensive studies of the interaction of glycopeptides with RCA I and RCA II are in general agreement with the results found for simple

saccharides (Debray 1981). A recent comprehensive review of the properties of lectins does not record any observations of binding of non-carbohydrates by RCA I (Goldstein 1986).

Inhibitor	Relative inhibitory potency	
	Agglutinin	Toxin
Galactose	1.0	1.0
Lactose	7.5	5.0
Melibiose	1.5	1.0
Raffinose	1.7	0.4
N-Acetyllactosamine	6	-
N-Acetylgalactosamine	<0.1	0.4
L-Rhamnose	2	0.2
D-Fucose	0.7	0.5
L-Arabinose	0.3	0.2
Methyl-galactoside	3.0	2.2
Methyl-galactoside	1.2	2.0
p-Nitrophenyl-galactoside	15	-
p-Nitrophenyl-galactoside	1.5	-
p-Nitrophenyl-N-acetyl galactosamine	1.7	-
Neu5Ac 2,3Gal 1,4Glc	4.0	-
Neu5Ac 2,6Gal 1,4Glc	16	-

From Goldstein 1986

Table 1. Binding of carbohydrates by RCA1

1.4.4 Lectins in the study of cells

Sugars constitute 2-10% of the mass of cell membranes, and the glycoproteins which they make up provide the cell with recognition patterns and give cells their individuality. Lectins are excellent probes for these cell surface components because they are specific, attach tightly and reversibly, and do not normally enter the cell. The simplest reaction of a lectin with cells is agglutination. However lectins can bind to cells without causing agglutination, and may cause other dramatic events in the life of a cell, for instance the stimulation of lymphocytes to undergo cell growth and division. This is known as mitogenis or lymphocyte transformation.

These actions of lectins may be inhibited by saccharides for which the lectin is specific. This may be taken as evidence that the receptor for the lectin contains a structure which is at least similar though not necessarily identical to that sugar. These saccharide receptors are components of membrane glycoproteins and glycolipids. Carbohydrate structures which strongly inhibit lectins appear to be a common occurence in a variety of glycoproteins. Such inhibitors of lectins are normally termed lectin receptors (Sharon 1975) although they do not necessarily communicate any signal to the inside of the cell.

It has been shown (eg Hammarstrom 1973) that association constants for the binding of lectins to cells are several orders of magnitude higher than those for binding the specific oligo- or monosaccharide for that lectin. This may be because the lectin receptors are more complex than the simple

oligosaccharides used in binding studies and lectins have a greater affinity for these extended sites, or lectins, being multivalent, may bind at more than one site. A third possible explanation is that non-specific interactions such as hydrophobic bonding may add to the strength of binding. It is well established that certain lectins do not compete for binding sites, indicating that their receptors are not only chemically different but also sufficiently far apart to prevent steric hinderance.

The presence of lectin receptors is easily demonstrated on cells by use of lectin derivatives, usually fluorescent or radiolabelled lectins. The methods used are generally similar to those developed by immunologists for the study of cell surface antigens. It is of course essential to ascertain that the binding is specific, eg it is inhibited or reversed by the appropriate saccharides. Fluorescent lectins may be visualised on intact cells in the light microscope under ultraviolet light. The amount of bound lectin may be determined by fluorimetry, or by measurement of radioactivity if radiolabelled lectins are used, and the data may be analysed by modifications of the Scatchard equation (Sharon 1975). A linear plot suggests a bimolecular reaction with a single equilibrium constant for all cell receptor interactions. A curvilinear plot of upward concavity suggests either a heterogenous receptor population or the existence of site - site interactions.

Lectins bind to the carbohydrate portions of glycoproteins and glycolipids that protude from cells. This conclusion is based on the observation that lectin binding is affected by

modification of cell surface sugars by glycosidases, glycosyltransferases, galactose oxidase and periodate. Similarly, treatment of cells with trypsin can cause a decrease of 35 - 50% in the number of lectin receptors (Kornfield 1969) presumably by removing the proteins to which they are attached. Unmasking of subterminal galactose residues of human lymphocytes or erythrocytes by enzymatic removal of sialic acid increases the amount of peanut agglutin bound by 20 to 40-fold (Carter 1977). Treatment of cells with neuraminidase increases binding of the galactose specific lectins RCA I and soya bean agglutin (Nicholson 1973) probably by revealing sites masked by neuraminic acid. Oxidation of galactose residues on the surface of human erythrocytes by galactose oxidase reduces the amount of soya bean agglutin bound by up to 90% (Lis 1982). In this case the receptors appear to be glycolipids. There is thus very good evidence for the specificity of lectins in binding to the carbohydrate portion of cell surface glycoproteins and glycolipids.
On both intact cells and isolated membranes lectin receptors are found on the outer and not the inner surface of the membrane (Nicholson 1978). This is consistent with findings obtained by other methods such as surface labelling with galactose oxidase and tritiated sodium borohydride, and provides strong evidence for the asymmetry of surface membranes. Asymmetric distribution of lectin receptors has also been found on intracellular membranes. There is increasing evidence that the binding sites are located on the cisternal (noncytoplasmic) side of many membranes, including

the nuclear envelope, the mitochondrial outer membane, chromaffin granules and the Golgi apparatus.

1.4.5 Lectin resistance

The cytotoxic properties and sugar specificities of lectins make them ideal for the study of cell variants with altered cell surface carbohydrates. Toxic lectins may be used to select cells with non-lethal defects of glycosylation. Cells are grown in the presence of concentrations of cytotoxic lectin that will kill most cells in the normal cell line but will permit resistant mutants to survive. Colonies of surviving cells may be cloned and in some of those clones acquisition of resistance may be accompanied by changes in the glycosylation of the cell surface. Such cells retain resistance to lectin even when cultured for several months in their absence and are therefore apparently stable mutants with low reversion rates. In many cases the biochemical basis for resistance has been found, for instance Hela cells resistant to ricin have an altered sialyltransferase, and mouse lymphoma and CHO cells resistant to PHA have an abberant GlcNAc transferase. Generally resistance correlates with decreased lectin binding capacity, although some mutants have unaltered binding. There are two ways in which cells may reduce their capacity to bind lectins: the sugar residues may be masked or sterically hindered, possibly by additional sugars, or they may be deleted from the oligosaccharide.

Both types of structural change have been found in resistant cells. Emergence of resistance may be the result of deletion of a single enzyme, but often seems to be the result of

changes in the activity of several glycosyltransferases. Masking of ricin receptors has been shown in baby hamster kidney and mouse L cells (Briles 1982 Stanley 1980). These variants contain much higher levels of sialyltransferase activity than wild type cells. The extra sialic acid residues are linked to and mask galactose residues that normally serve as ricin binding sites.

Oligosaccharide deletions have been found for a number of sugars and affecting cells to varying degrees. In a wheat germ agglutin (WGA) resistant CHO line the cells have a sialic acid deficiency in their oligosaccharides (Briles 1981). Such deletions may range from almost complete absence of sialic acid to a deficiency only of sialic acid residues with specific linkages, for instance 2 - 6 to galactose.

These variants have a decreased ability to bind wheat germ agglutin but an increased number of terminal galactose residues to which ricin may bind, which explains the hypersensitivity of these variants to ricin. Interestingly these cells appear to possess all the elements necessary for sialylation, namely the sugar nucleotide donor, acceptor and transferases (both 2 - 3 and 2 - 6) since extracts of these cells are capable of carrying out sialylation to the same extent as wild type cells. This suggests that the defect is somewhere in the intracellular organisation of protein biosynthesis (Briles 1982).

Variants of mouse lymphoma cells have an almost complete absence of fucose (Briles 1982 Reitman 1980). These variants are specifically deficient in GDP mannose - 4,6 -dehydratase, an enzyme that participates in the conversion of GDP mannose

to GDP - L - fucose. Fucose is required for pea lectin to bind, hence the resistance of these cells to pea lectin. A number of interesting variants have been found which are deficient in both sialic acid and galactose. The sialic acid deficiency is presumably secondary to the galactose deficiency, since sialic acid normally follows galactose in oligosaccharide chains. In these cells glycolipids appear to be affected as well as glycoproteins. One of the best researched of these is a CHO variant called clone 13 which contains only 12-15% of wild type levels of galactose and sialic acid in its membranes and shows much reduced binding of WGA and ricin compared to wild type cells (Briles 1981). Clone 13 contains normal levels of UDPgalactose, suggesting that impaired galactose synthesis cannot account for the defect. Three other galactosylation defective variants have been selected from mouse lymphoma cells, Swiss mouse 3T3 cells and CHO cells. In these experiments PHA, GS I and abrin respectively were used as the cytotoxic agents (Briles 1982). The mouse lymphoma variant fails to add galactose to its Asn-linked complex oligosaccharides as shown by labelling studies and glycopeptide analysis, even though in vitro its galactosyl transferase and epimerase appear to be normal (Trowbridge 1978). In the GS I resistant variant, a similar failure to galactosylate has not been shown directly, but may be inferred from cytotoxicity and lectin binding studies which show a greatly reduced binding of both ricin and GS I, but increased binding and sensitivity to GS II, a lectin which is specific for terminal non-reducing N-acetyl D-glucosamine groups (Stanley 1979). A similar lectin binding pattern is

observed with the abrin resistant CHO line described by Li et al (1980). As with clone 13 no galactosyl transferase deficiencies were found in these variants. Since these lines contain the elements necessary for galactoside biosynthesis it is likely that the defect involves intracellular organisation, as with the sialylation defect described above.

More extensive deletions have been found in resistant cells that lack the entire outer branch of complex or N-acetyllactosamine type glycosidic units, consisting of sialic acid, galactose and N-acetylglucosamine (Briles 1982 Stanley 1980). These variants show decreased binding of lectins binding to outer branches, eg WGA, ricin, PHA and lentil lectin. They have an increased mannose content and are therefore more sensitve to conA cytotoxicity than wild type cells.

A ricin resistant mutant of baby hamster kidney cells had an accumulation of oligomannose glycans (Hughes 1983). Sialylated N-acetyl lactosamine type glycans were either absent or greatly reduced. Interestingly, these alterations were expressed in fibronectin, a secreted glycoprotein, as well as cell surface glycoproteins.

In all the variants discussed above the structural alterations are in the N-acetyllactosamine chains of glycoproteins. Another class of variants have been produced by selection for resistance to conA in which deletions occur in the oligomannose units (Briles 1982).

As well as helping investigation into the biosynthesis of carbohydrate chains lectin resistant mutants provide insight into the biologocal role of cell surface sugars. A ricin

resistant mutant derived from canine kidney cells is unable to incorporate galactose or sialic acid. However it retains the structural polarity characteristic of epithelial cells and the ability to develope tight junctions (Meiss 1982). In contrast to this lack of consequence of an oligosaccharide deletion, a rat L6 myoblast cell line resistant to conA was unable to undergo normal cellular differentiation to form myotubes. A high correlation was found between lectin resistance and loss of fusion potential, indicating that the two properties were probably related (Parfett 1981). No variant has been reported that is more than three times more resisitant than wild type cells to conA: more drastic alterations decreasing conA binding further appear to be lethal to cells.
It is however clear that a number of variants with severly truncated glycoprotein chains can be isolated. This is compelling evidence that all complex chains are not essential for cell viability.

1.5. Aim of this work.

Techniques of tissue culture have not been very extensively applied to inherited diseases of galactose metabolism and the effect of epimerase deficiency on cells is not known. Cell culture media can affect the metabolism of cells with enzyme disorders in many ways but this has not been considered with respect to disorders of galactose metabolism. The toxicity of galactose on cells lacking epimerase is not known. Whilst lectins have been used to produce mutant cells lacking certain enzymes and to study the effect of that lack on glycoproteins, they have not been used to investigate the effects of

inherited diseases of galactose metabolism on glycoproteins.

The aims of this project are therefore:

1. To examine the galactose content of tissue culture media with a view to producing an environment with a known galactose content for growing cells.
2. To examine the ability of epimerase deficient cells to grow without galactose and to establish the existence and extent of any requirement for galactose supplementation that they may have.
3. To investigate the applications of lectins to the investigation of inherited diseases of galactose metabolism.
4. To establish whether galactose is toxic to epimerase deficient cells.
5. To determine whether epimerase and transferase deficient fibroblasts can produce glycoprotein of normal carbohydrate composition.

Chapter 2

MATERIALS

All cell culture materials including tissue culture vessels, culture media and foetal calf serum were obtained from Gibco Ltd., Trident House, Renfrew Road, Paisley, Scotland. All cell lines were from the stocks held at the South West Regional Cytogenetics Laboratory, Southmead Hospital, Bristol except both Chinese Hamster Ovary cell lines which were a gift of Professor Monty Krieger, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA, and human fibroblasts line ED2, given by Professor Luigi Garibaldi, Department of Pediatrics, Institutio G. Gaslini, Genoa, Italy.

Bulk liquids including all solvents, aqueous acids and Cocktail T scintillant were 'AnalaR' grade and came for BDH Chemicals, Ferris and Company Ltd., Kenn Road, Bristol.

Isotopically labelled compounds were produced by New England Nuclear and supplied by Du Pont (UK) Ltd. of Southampton. The carbon-14 galactose used had the isotopic labelling distributed throughout the carbon skeleton. All other chemicals, reagents, enzymes, lectins and smaller items of equipment including dialysis tubing and filters were purchased from Sigma chemical Co. Ltd., Fancy Road, Poole, Dorset.

The following pieces of equipment were used:

A laminar downflow recirculating sterile work station from MDH Ltd.

A K1 thermostatic compact incubation (LEEC Ltd.).

A 35 HL Liquid Nitrogen refrigerator (Union Carbide Ltd.).

A Zeiss standard 3 objective microscope and a Prior monocular inverted microscope.

A Shimadzu fluorimeter.

An LKB 1210 Ultrabeta Liquid Scintillation Counter

Pye Unicam SP1800 spectrometer.

Chapter 3

METHODS

Section 3.1 Cell Culture

3.1.1 Sterility

Cell culture was carried out by conventional techniques for the growth of human fibroblasts and Chinese Hamster Ovary Cells (CHO cells). Normal precautions were taken to ensure that solutions and equipment did not become contaminated with micro-organisms. In particular, all work with open solutions or culture vessels was carried out in a laminar downflow work station. Solutions which required sterilisation were either autoclaved at 121°C for 15 minutes or were filter sterilised. Small volumes (less than 50ml) were filtered through Millex GV filters, larger volumes (up to about 200 mls) through Sterivex-GS filters and volumes greater than this through Falcon 7103 filtration units.

3.1.2 Establishing Cultures

Skin biopsies were taken by clinicians from children who were known to be affected with relevant inherited diseases. Biopsies were taken according to the method of Edwards. The forearm or iliac crest was used. The surface of the skin was cleaned with ether and allowed to dry. A piece of skin was pinched between forceps and the top sliced off with a scalpel to a sufficient depth to include the whole thickness of the epidermis. The biopsies were transported to the laboratory in normal growth medium in a sterile container. The biopsy was then dissected with forceps into pieces approximately 1 mm cubed. The pieces were divided between two tissue culture flasks and anchored in place by plasma clots. For this 0.1 ml $CaCl_2$ (20 g/L) solution was added to 0.5 ml of

human plasma. A few drops of this recalcified plasma was placed on each explant and left to clot. Five mls of warmed growth medium was then added to each flask. The growth medium was changed every 2 days until sufficient fibroblasts had grown from the explant to allow subculture.

3.1.3 Subculture

Cells were removed from flasks containing explants or cell monolayers with versene/trypsin solution (VT). The medium was discarded and the flask washed with warmed PBS. This was discarded and replaced with VT, 5 mls for small flasks and 12 mls for large flasks. The flask was then incubated until cells were rounded and free from the flask surface. The cell suspension was then centrifuged in a bench top centrifuge at 1000 rpm for 5 minutes. The supernatant was then discarded and the cells resuspended in growth medium. This was divided between culture vessels as required with a split ratio of 1:2 or 1:3. Growth medium was then added to a level of approximately 0.2 mls/square cm of culture area. Subculture number was recorded each time and cells were not used past subculture number 15.

3.1.4 Freezing

Cells were frozen using dimethyl sulphoxide (DMSO) as a cryoprotectant. Three ampoules could be produced from every 75 cm^2 flask. Cells were trypsinised as for sub-culturing but the cells were resuspended in medium containing 5% DMSO in the amount of 3 mls of medium for every 75 cm flask, and if more was used then all cells of each line were pooled. The

cell count was determined and 1 ml of suspension dispensed into each ampoule. Ampoules were then labelled and a record card written recording the name, disorder, subculture number and cell count. The cells were frozen in a plasma cell freezer at a rate of -1°C per minute to -30°C then -10°C per minute to -190°C. The frozen cells were then transfered to the liquid nitrogen storage bank for long term storage.

3.1.5 Counting cells.

Cells were counted in an Improved Neubauer counting chamber. A drop of cell suspension was put into the counting chamber which was then examined under a microscope. The cells in four large (1 mm x 1 mm) squares were counted with the aid of a tally counter and the average number per square found. This was multiplied by 10000 to give the number of cells per ml.

3.1.6 Recovering cells from liquid nitrogen.

Ampoules were removed from liquid nitrogen and quickly placed in a 37°C water bath. When thawed, the contents were transferred to a $25cm^2$ culture flask and 10 mls of medium added then the flasks were placed in a 37°C incubator. The cells were observed after one or two hours to ensure that they were adhering to the bottom of the flask. The next day the medium contaminated with the DMSO used in freezing was discarded and replaced with 5 mls of fresh growth medium.

3.1.7 Culture vessels.

A number of different culture vessels were used;the size of vessel chosen depended on the number of cells required for an experiment and the number of replicates in which it was carried out. The smallest area for use if large numbers of replicates were required was a 2 cm x 2 cm square well of a 25 well plate. Next in size were 6 cm ambitubes and Lux 9 cm 8 well plates, then conventional tissue culture flasks of 25cm^2, 75cm^2 and 200cm^2.

3.1.8 Mycoplasma testing.

A washed sterile 22 x 22 mm glass coverslip was placed in a labelled well of a Lux multiplate for each line to be tested. One ml of cell suspension containing about 0.25 to 0.5 million cells was run onto each coverslip. The plate was then incubated at 37°C for 24 h . The medium was then removed and the well washed with 2-3 ml of PBS which was discarded. Two to three mls of Carnoy's fixative was then added to each well and left for 5 min. This was removed and the fixation repeated for an additional 10 mins. Coverslips were then air dried for 30 mins. The coverslips, along with a fixed infected control slide, were then stained for mycoplasma. One ml of Hoechst 33258 stain solution was added to each well and left for 30 mins at room temperature.

The stain was pipetted off and the coverslips washed in distilled water. They were then blotted dry and a drop of mycoplasma mounting medium placed on the upper cell covered surface. A labelled slide was then placed on top, pressed to expel excess solution and blotted dry. The edges of the

coverslip were then sealed with clear nail polish.
Slides were examined in a Leitz fluorescence microscope under UV illumination. Cells were located with a x 12.5 objective and screened for mycoplasma with a x 100 oil objective.
The affected control slide was examined at the same time.

3.1.9 Control cell lines.

Normal control cell lines were acquired from the cell bank in the Department of Cytogenetics, Southmead Hospital, Bristol. Lines chosen were either those donated by apparently normal volunteers, or those referred to the Department of Cytogenetics for chromosomal analysis and found to be normal chromosomally. Samples referred for any sort of biochemical analysis or those from a patient with a history suggestive of a biochemical disorder were not included.

3.1.10 Maintainance Culture.

While cells were being grown in culture vessels, the medium was changed every Monday, Wednesday and Friday. The amount of medium used was approximately 0.2 ml per square cm of culture area. The medium was warmed to 37° before being added to the culture vessel.

Section 3.2: Experiments on the Galactose Content of Cell Culture Media

3.2.1 Estimation of the galactose content of cell culture media and sera by gas chromatography/mass spectrometry.

Deproteinised, derivatised samples of cell culture media and sera were subjected to carbohydrate analysis by gas chromatography/mass spectrometry. The method used was based on that for the gas chromatographic measurement of blood and urine glucose and other monosaccharides by Murphy and Pennock (1972). Deproteinisation and derivatisation were carried out substantially as described in this reference. The conditions for chromatography were adapted from those for a packed column to those suitable for a capillary column, and flame ionisation detection replaced with mass spectrometric detection. For this, various experiments were carried out to discover the best ions to measure. The method used was as follows. Deproteinisation was carried out by the method of Somogyi as described in the reference, except that samples were not diluted with 3.4 ml of water. To 250μl of the solution under test 50μl of mannitol solution was added as an internal standard, followed by 500μl of barium hydroxide solution. The mixture was vortexed and 500 μl of zinc sulphate solution added. The samples were vortexed, centrifuged at 3000 rpm for 5 minutes then 500μl of supernatant decanted. This was dried down in a glass conical tube at 70°C under a stream of nitrogen. Derivatisation was then carried out to form a methoxylamine acetylated derivative for gas chromatographic analysis. One hundred microlitres of methoxylamine in pyridine was added to each sample and after 15 minutes

incubation at 30°C 100μl of acetic anhydride was added and the incubation continued for a further 15 minutes. Derivatives were then taken to dryness under a stream of nitrogen, again at 30°C. One hundred microlitres of both chloroform and water were added to each sample, and after vortexing the aqueous layer was removed.

The conditions used for chromatography were: split ratio 50:1 with one ml per minute of sample flow through column and 30mls per minute of carrier flow. Injection temperature was 230°C and detector 200°C. The column used was a Chrompack WCOT 12 meter fused silica. The instrument was set up to record the total abundance of all ions between 100 and 600 atomic mass units and the abundance of a single designated ion, usually 187.0 amu. Mass spectra were recorded of all significant peaks, and single ions chosen on the basis of spectra content.

3.2.2 Determination of conditions for dialysis

Foetal calf serum was dialysed to remove uncombined galactose that it might contain. To establish the dialysis conditions which effectively removed galactose, 1μl of radiolabelled galactose solution was added to 40 ml of foetal calf serum. This was sealed in a dialysis sack which was placed in 2L of saline. One ml of saline was removed every hour for 8 hrs, mixed with 20 ml of cocktail T scintillant and the radioactivity measured in a beta-counter.

3.2.3 Dialysis of serum

Conditions for routinely dialysing serum were established as a result of the experiments described in section 3.2.2. For

routine dialysis, 40 ml of foetal calf serum were placed in a dialysis sack and this was sealed and immersed in 2L of saline for a minimum of 6 hrs and maintained at a temperature of 4 degrees Celsius . The saline was then discarded and replaced with 2L of fresh saline and dialysis continued for a minimum of a further 6 hrs. This was repeated once more to give three changes of saline. The foetal calf serum was removed from the sack, filter sterilised and stored at 4 degrees centigrade until used, or for a maximum of 2 weeks.

3.2.4 Solid Phase Linked Lectins

Affinity chromatography with solid phase linked ricin lectin (SPLL) was used to separate proteins with exposed galactose residues from those lacking them. Columns of ricin lectin insolubulised on cross-linked 4% beaded agarose gel were used. An empty econo-column was filled with gel to a packed volume of 1 ml. One ml of the solution under test was gently run onto the column and eluted with 1 ml aliquots of either 1 M aqueous NaCl or 1 M aqueous NaCl containing 0.1 M galactose. The latter solution displaced compounds adhering to the column by their galactose residues, allowing them to be collected and the columns to be regenerated. All eluant was collected in 1 ml aliquots. After use the columns were washed with 20 mls of 1 M aqueous NaCl to remove all galactose. When not in use the columns were filled with sufficient 1 M NaCl solution to cover the gel and stored at 4°C.

3.2.5 Protein analysis - Folin and Ciocalteau.

Two variants of this method were used. In some experiments parameters were determined for populations of growing cells and related to the amount of protein present by solubilizing the cells in 0.1 M NaOH and measuring the amount of protein in this solution. For this protocol, standards of bovine serum albumin(BSA) were made up in the range 25 to 200 mg/l and stored deep frozen. Solutions of 10 g/l $CuSO_4$ $5H_2O$ and 20 g/l sodium tartrate were made up, stored separately and equal volumes mixed immediately before use. One part of this solution was mixed with fifty parts of sodium carbonate (20g/l in 0.1 M NaOH) to form solution A. Folin and Ciocalteau reagent was diluted with an equal volume of water to form solution B. To 200 μl duplicates of sample, standard or blank (0.1 M NaOH) 1 ml of solution A was added, the solutions vortexed and left for 10 minutes at room temperature. Then 0.1 ml of solution B was added and the mixture vortexed. After a further 30 mins, the absorbance at 750 nm was measured in a spectrophotometer.

The second variant was used for measuring the protein content of solutions on which Western Blots were done. Standards between 100 and 500mg/l of BSA were made in SDS (10g/l) in aqueous NaCl (9g/l). For this protocol solution C consisted of 1g/l of $CuSO_4$ $5H_2O$ and 2g/L of sodium tartrate, and solution D of 8 g/l of NaOH and 21.2 g/l of Na_2CO_3. Ten mls of sample were diluted with 190μls of 1 g/l SDS solution. Fifty microlites of this dilution was added to 0.4 ml of solution C and 0.2 ml of solution D in duplicate, the mixture vortexed and incubated at room temperature for 10 minutes.

Then 50μl of a 1 in 5 dilution of Folin & Ciocalteau reagent were added, the mixture vortexed and incubated for 30 mins at room temperature, and the absorbance again determined at 750 nm.

3.2.6 Protein Analysis Coumassie Brilliant Blue Dye Binding

The method used was essentially that of Sedmak and Grossberg. The dye solution (60mg/l) was prepared in 0.6 M HCl, then filtered to remove undissolved material, and stored at room temperature. Standards of BSA were prepared in the range 25 to 500mg/l in the same solution as test samples had been dissolved in. For the protein assay 500μl of dye solution was added to 500μl duplicates of protein solution, mixed immediately and the absorbance at 620 nm determined against a 1:1 mixture of blank and dye. In some assays the volume of protein solution was reduced to 250 or 125μl if the protein had been dissolved in alkaline since this assay requires a strongly acid environment. Standard curves were run at various ratios of dye to protein solutions to establish which ratio produced the optimum standard curve.

3.2.7 Assays of epimerase in cultured cells

This assay was carried out on cell homogenates by the method of Gillett (1985). Cells growing in flasks were trysinised, a small aliquot was counted and the remaining suspension certrifuged. The supernatant was discarded and the cells resuspended in PBS and centrifuged again. This washing procedure was repeated twice more. After the final supernatant was discarded the cells were suspended in

sufficient distilled water to give a count of approximately 10^7 cells per ml. The suspension was frozen and thawed 3 times by alternate immersion in liquid nitrogen and 37° water bath then centrifuged to remove cell wall debris. The supernatant was stored in liquid nitrogen until required for assay.

The epimerase assay was carried out in duplicate in small polythene test tubes. To each tube was added 10μl of 6mM NAD, 10μl of water, 20μl of buffer 10μl cell lysate and 10μl of radiolabelled UDP gal, 2mM, 1.2 μCi/ml. The buffer was made from 4.5g glycine and 15.4 mg dithiothreitol brought to pH 8.9 and made up to 100 ml with water. For determining the epimerase activity of serum, 10 μl FCS was used in place of the lysate. The mixture was incubated for one hour at 37°C and for each duplicate a blank was run for which the lysate had been boiled to remove any enzyme activity. The reaction was stopped by placing the tubes in a boiling water bath for 2 minutes. A polyethylene-immune cellulose plate was prepared by running in a chromatography tank containing a small amount of distilled water until the solvent front reached the top of the plate, then drying in a warm air oven. Five μl of each reaction mixture were seeded on a 1cm line 1.5 cm above the bottom of the plate. The plate was then run in a solvent consisting of 13.8g of boric acid, 8.8 ml of tetramethylamine and 30ml ethanediol made up to 200ml with distilled water. When the solvent front had almost reached the top of the plate, it was removed from the tank and dried. It was then scanned with the Berthold scanner to detect the UDPgal and UDPglu peaks. These were cut out, placed in a scintillation

vial, covered with scintillation fluid and the amount of radioactivity determined in a β-counter.

Protein concentrations in the lysates were determined by the method of Folin and Ciocalteau and the epimerase activity expressed for each sample as n moles converted per mg of protein per hour.

Section 3.3 - Normal Growth Rates of Cells

3.3.1 Determination of rates of incorporation of radio labelled leucine, uridine and thymidine.

These experiments were carried out in 5x5 multiwell plates with each well having an area of 4 cm^2. Cells were subcultured from normal culture vessels to multiwell plates and grown to a density of 25 to 50% of confluence as judged when examined by inverted microscope.

For experiments on the leucine incorporation rate, the medium in test wells was replaced with 0.5ml medium containing 1 μci/ml of ^3H leucine and the plate incubated at 37° for 1 hour. At the end of the incubation period medium was removed from blank wells and replaced with medium containing labelled leucine. Medium was then quickly removed from all wells, each well washed four times with PBS and 200μl of VT solution added to each well.

When the cells were rounded up, 100μl was removed from each well and run onto a fibreglass filter paper disc in a Millipore vacuum filtration system. The disc was washed by running 10ml of 10% (W/V) TCA in water through the system, followed by another 10ml of TCA solution and finally 10ml of IMS alcohol. The filter disc was removed, placed in an open scintillation vial and allowed to dry. Two ml of scintillation fluid were added to each vial and the radioactivity measured in a β-counter. To determine the amount of protein in each well, 250μl of 0.1 M NaOH was added to the remaining 100μl of cell suspension. Duplicate aliquots of 150 μl of this mixture were removed for protein measurement by the Coumassie Brilliant Blue method.

The resulting activity was expressed as counts per minute per unit protein. For measurement of the uridine incorporation rate, cell culture medium was supplemented with ^{3}H uridine to a level of 1 μCi per ml. The procedure was the same as for leucine, except that the cells were incubated for 6 hours at 37° before the medium was removed.

Similarly, for thymidine incorporation, cell culture medium was supplemented with ^{3}H thymidine to 1 μCi per ml, and cells were incubated in this medium for 24 hours, otherwise the protocol was the same as for leucine.

3.3.2. Comparative growth rates of cell lines.

These experiments measured the increase in cell numbers under normal culture conditions for various cell lines. The lines used were transferase deficient, epimerase deficient and normal control human fibroblasts, and epimerase deficient and normal control CHO cells.

A confluent 25cm^2 flask was produced for each cell line used. Cells were removed from the flask surface with VT solution as described in section 3.1.3. Five mls of cell suspension was produced from each flask and this was plated out at a rate of 0.2 ml in each of 25 wells of a multiwell plate, and the cells were incubated for 24 hours to allow recovery from trypsinisation and re-adhesion.

The cells in four wells were then counted by washing the wells with PBS, adding 200 ml of VT solution and counting the suspended cells in a modified Neubauer counting chamber as described in section 3.1.5. The counted cells were discarded, and the remaining wells were incubated further. Subsequent

quadruplicate counts were made after 48, 96 and 168 hours.

Section 3.4 - Growth Rates in Media With Added Galactose

3.4.1 Incubation of cells with Galactose.

A small flask of cells from each line under investigation was grown almost to confluence. The cells were then trypsinised, suspended in PBS and a small aliquot was counted. The suspension was divided equally between wells of the multiwell plate to give about 25000 cells in each $4cm^2$ well. One ml of medium was then added to each well and the cells incubated for 24 hours to allow them to adhere and recover from trypsinisation.

Medium supplemented with galactose to a level of 1mM was then prepared by dissolving the appropriate amount of galactose in medium and filter sterilising. Five wells for each cell line were trypsinised with 200 μl of VT solution and the cells counted to give a value for the cell density at time zero. The medium in all other wells was replaced with galactose supplemented medium. The medium was replaced every 2 or 3 days as in normal maintenance cultures. After 24 hours, 6 days and 10 days, a further five wells for each line were trypsinised and counted.

3.4.2 Photography of cells incubated with galactose.

For each line under investigation a small flask of about 25% confluence was prepared. The medium in the flask was replaced with medium containing 1mM galactose prepared as described in section 3.4.1. Photographs of the cells were taken using an inverted microscope with camera attachment on black and white film at the start of the experiment, and after 6 and 10 days growth in the galactose containing medium. The medium was replaced every 2 or 3 days as in normal maintainance culture.

3.4.3 Incubation of cells with radiolabelled galactose.

These experiments attempted to establish the rate of production of ^{14}C labelled CO_2 from ^{14}C labelled galactose. Cells were incubated in a variety of media containing different concentrations of glucose, galactose and other additives and were incubated for a range of times.
The cell lines under test were grown in normal tissue culture vessels then subcultured into Erlenmyer flasks containing approximately 4ml of medium, and allowed to grow for at least 24 hours before incubation with labelled galactose was started.
The media used were normal TC199 supplemented with foetal calf serum and glutamine, glucose free TC199 with foetal calf serum and glutamine in some experiments with galactose added to a concentration of 1 or 5mM, PBS and Krebs-Ringer buffer. The amount of radiolabelled galactose used were 0.05 and 0.1 μCi per ml of culture medium. In some experiments lactic acid was added to the medium to a concentration of 0.7 mM. The period

of incubation of labelled galactose ranged from 2 hours to 4 days.

For each flask used a filter paper disc was prepared from Whatmans No 1 filter paper of the same diameter as the inside of the cap of the flask. One drop of concentrated NaOH was put on this and the disc placed inside the cap. The medium in the flask was discarded and replaced with the chosen medium containing radiolabelled galactose. Two mls of this medium were run in from a sterile pipette, taking care that no medium touched the neck of the flask. The flasks were then incubated at 37° for the length of the experiment. At the end of the experiment the caps were removed, 100 μl of 1M $HClO_4$ was run into each flask, the caps quickly replaced and the flasks left for 1 hour. The filter discs were then taken out, placed in scintillation vials containing 2ml of cocktail T scintillant and the radio-activity counted in a β-counter. Blanks were run using Erlenmeyer flasks to which no cells had been added but which were treated the same. For some experiments cells were grown in the glucose-free medium supplemented with 5 mm galactose, for 24 hours before the experiment.

Section 3.5: Combined Galactose in Cells

3.5.1 Western Blot: Gel Electrophoresis.

The presence of exposed galactose residues in soluble fibroblast proteins was investigated by Western Blotting, i.e. solubilisation of cell proteins and gel electrophoresis followed by transfer of the electrophoresed protein to cellulose acetate and detection with a lectin/enzyme linked antilectin antibody system.

Cell lines under investigation were grown to approximate confluence in two large cell culture flasks. The cells were trypsinised off, suspended in PBS and counted. The cells were pelleted by centrifugation and the PBS decanted. Cells were solubulised by the method of Anderson and Giehmberg (1978). Approximately 10^7 cells per ml were dissolved in PBS containing 1% (w/v) Triton X-100 and 2mM phenylmethylsulphonyl fluoride at 4°C, and the mixture centrifuged at 3000g for 10 minutes at 4°C. The supernatant was decanted and 50 μl removed for protein estimation by the method of Folin and Ciocalteau. The remainder of the supernatant was diluted with an equal volume of sample buffer containing 5% mercaptoethanol and stored deep frozen until required.

SDS - polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (1970) with acrylamide concentrations of 3% (w/v) for the stacking gel and 8% (w/v) for the separating gels. The gel size was 150mm x 120mm x 1mm. Solubilised erythrocytes (150 μg) duplicates in adjacent wells and molecular weight markers were electrophoresed for each experiment. Electrophoresis was carried out at 30V per slab for 16-24 hours, until the tracking dyes had almost reached the bottom of the gel.

Western Blotting - electrotransfer.

This procedure was performed essentially according to the method of Towbin (1979). The SDS gel was removed from its glass plates and a piece of cellose nitrate paper cut to the same size as the gel placed on top. A sandwich was formed consisting of one piece of Scotchbrite and 2 pieces of filter paper both soaked in transfer buffer on either side of the gel

and this was placed in a transfer cassette. This was then placed in a transfer tank and electrophoresed in a plane and perpendicular to the sandwich for 20 hours at 250 mA and 4°C. After transfer, the gel and paper were separated. The gel was fixed in 250 mls of 50% methanol, stained for 30 mins with Coumassie Brilliant Blue (CBB) then destained with GAA/methanol/water destaining solution. The nitrocellulose membrane was cut into strips (16 x 130 mm) corresponding to the gel slots. Protein on the molecular weight strips was visualised by staining with amido black solution, then destained with the same solution as was used for CBB. Non-specific protein binding on the nitrocellulose was blocked by soaking the strips in skimmed milk powder solution (5g in 100 mls of PBS/tween) for 30 mins. then rinsing 3 times with PBS. The pairs of strips for each sample were divided into a test strips and a control strips and each set placed in a shallow plastic tray. For detection of galactose in the membrane bound proteins, the test strips were incubated with galactose specific lectin (control strips with lectin plus galactose), then all strips were incubated with rabbit anti-lectin antibody followed by peroxidase-linked anti-rabbit antibody plus a chromogen producing colour development at the site of lectin binding. Specifically, test strips were incubated for 1 hour at room temperature with 5ml of PBS/tween containing 75 ml of lectin concentrate, control strips were incubated under the same conditions with 5ml of lectin and PBS/tween solution to which galactose had been added to a concentration of 0.3M.

Test strips were washed three times with 50ml of PBS and control strips with 50ml of PBS/galactose. Each set of strips was then incubated with 7.5ml anti ricin antibody in PBS plus milk powder for 1 hour. The antibody solution was removed and strips washed 6 times in PBS with a 5 minute soak for every wash. Each set of strips was then incubated with 5ml of peroxidase-conjugated swine anti-rabbit antibody in PBS plus skimmed milk powder for 1 hour. The antibody was decanted and the strips washed 6 times in PBS as before.

Antibody binding was revealed by incubating the strips with diaminobenzidine in PBS containing 0.02% (v/v) hydrogen peroxide until bands became visible (between 10 seconds and 1 minute). The reaction was terminated by washing the membranes for 5 minutes in distilled water. Membranes were then dried on filter paper and stored in the dark.

Upon completion of the experiment, membranes and the polyacrylamide gel were photographed by the Medical Photography Department, Southmead Hospital.

3.5.2 - Staining Cells with Fluorescein Isothyocyanate-linked Lectin.

Fluorescein isothiocyanate (FITC) linked ricin is a sensitive and specific stain for exposed galactose residues. It was used in this experiment to stain a range of living cells. Sterile, acid washed coverslips (22 x 22 mm) were placed in each well of an 8-well plate. A suspension containing approximately 10^5 cells in 1 ml of medium was run onto the coverslips and the cells incubated for 24 hours, on a slight incline to give a range of cell densities across the slide.

Coverslips were removed from the wells, washed three times with 2ml of PBS then each coverslip was cut in half with a diamond tipped scriber. The two halves were put in separate wells of a multiwell plate. Two hundred microlitres of fluoroscein-linked lectin solution (50 μg per ml in PBS with trypan blue) were run onto one half of each coverslip and 200 μl of lectin solutions plus galactose (50 mM) onto the other. The coverslips were incubated for 30 mins then the lectin containing solution removed and each well washed 5 times with 10 ml of PBS. Coverslips were mounted cell surface downwards on glass slides with a drop of PBS and the edges sealed with clear nail polish. Slides were examined immediately under phase contrast and UV illumination using x25 and x40 objectives. The slides were photographed using the camera attachment for the microscope under phase contrast and UV illumination by automatic exposure with spot metering centred on a fluoresing cell. The results of visual examination were also recorded as the presence or absence of fluorescence together with an estimation of the relative degrees of staining of similar cell lines.

3.5.3 - Quantitation of Fluorescein Isothyocyanate Binding by Fluorimeter.

For each cell line under investigation a twenty-five well plate was used. Cells were grown in medium containing dialysed serum, medium made with non-dialysed serum or medium supplemented with galactose, for 2 weeks before the experiment. Between 50,000 and 200,000 cells in 200 μl of medium were put in each 4cm^2 well, giving a range of cell

densities in each plate, and the plates were incubated for 24 hours to allow the cells to adhere and recover from trypsinisation. The medium was removed and the wells rinsed 3 times with 2ml of PBS. To 20 of the wells on each plate, 1 ml of PBS containing FITC linked lectin (20 ng lectin protein/ml) was added. The other 5 wells were designated blanks, and to these 1 ml of lectin in PBS plus galactose (50 mM) was added. The plate was incubated for 30 minutes at room temperature then the lectin containing solutions removed and each well washed 5 times with 5ml of PBS. One ml of 0.1 M NaOH was added to each well to dissolve the cells. Four hundred microlitres were removed and used for protein estimation by the Commassie Brilliant Blue method. One ml of water was added to each well, the contents removed and placed in a fluorimeter cell. In some experiments the cells were counted before being dissolved in NaOH. For this, 200 μl of VT solution were added to each well, the cell count determined by used of a counting chamber, then 1 ml of 0.1 M NaOH added to each well, and the contents of the well placed in a fluorimeter cell.

The fluorescence was determined in the Shimadzu fluorimeter. The settings used were: excitation wavelength 495 nm, (bandwidth 3nm), emission wavelength 515 nm, (bandwidth 5nm) and averaging time 1 sec. The instrument was set to zero with 0.1 M NaOH in the fluorimeter cell then the gain adjusted to give a reading of 50 units when the fluorescence standard was placed in the cell. Initially, a sample was scanned over a range of excitation and emission wavelengths to confirm the

choice of wavelengths. Samples were then read, with the blank and standard being checked frequently to avoid instrumental drift.

The fluorescence per unit protein or per 1000 cells was then determined.

3.5.4 - Determination of carbohydrate composition of cells by gas chromatography.

The concentrations of a number of monosaccharides were determined in hydrolysates of whole cells. This was done by gas chromatography using the method of Bhatti, Chambers and Clamp (1970).

Cells were grown for three subcultures in media containing dialysed serum or undialysed serum, or medium supplemented with galactose to 1 or 5 mM. Each cell line under investigation was grown to confluence in a large flask using the chosen medium. The medium was removed and the flask rinsed three times with PBS. The cells were trypsinised off with 15ml of VT, spun down and resuspended in 1 ml of PBS. Aliquots of 500μl of this suspension transferred to small glass ampoules. These were centrifuged at 3000 rpm for 5 minutes and the supernatant decanted, 0.5ml of water was then added to each ampoule and they were vortex mixed. Fifty μl was removed for protein analysis by the Coumassie Brilliant Blue method and the remainder was frozen in liquid nitrogen then lyophilised.

The technique for gas chromatography were as described in the reference (Bhatti 1970). In essence 0.5 ml of methanolic HCl (1.5 M) and mannitol as an internal standard were added to

each ampoule. They were sealed and incubated at 90°C for 24 hrs, after which the acid was neutralised by the addition of solid silver carbonate. The supernatant was transferred to a pear shaped flask and evaporated under reduced pressure. Acetic anhydride (0.1ml) was added and the flasks incubated at room temperature for a minimum of 6 hrs. Silylating reagent (50μl) was added and the flasks left for 30 mins. After centrifugation, 1-5μl of supernatant was injected into the gas chromatograph. An SE 30 column was used with a temperature programme of 140°C to 200°C at 0.5°C/minute. Amounts of monosaccharides in the sample were calculated by integration of sample and internal standard peaks and the amounts of monosaccharides per unit protein were calculated from peak areas and the previously measured protein values.

Chapter 4

RESULTS AND DISCUSSION

4.1 Cell Culture.

4.1.1 Culture viability.

All cell lines chosen including those from patients with inherited metabolic diseases were successfully cultured, grew at adequate rates and were able to progress to confluent growth under the culture conditions described.

4.1.2 Infection

Two episodes of bacterial infection of culture medium occurred during this work. Both were immediately obvious: the excess metabolic activity of the bacteria caused the pH indicator in the TC199 medium to become bright yellow, showing an abnormally low pH, and bacteria were visible on microscopy. Contaminated cultures were discarded.

4.1.3 Mycoplasma Testing.

No intracellular fluorescence was observed on any slide prepared from cells cultured during the project. The fixed infected control slides showed punctate intracellular fluorescence on all occasions that the test was carried out. These results suggest that all cultures remained mycoplasma free for the period of experimentation.

4.1.4 Cryopreservation.

Recovery of frozen cells from liquid nitrogen storage produced live cells capable of adhesion and division on all occasions.

4.1.5 Karyotypes.

All human fibroblast lines had diploid chromosomes which were normal in number and visible structure on microscopy, and which were appropriate for the sex of their origin. This normality was preserved when the epimerase deficient cell line was tested during and at the end of the experimental period.

4.1.6 Confirmation of enzyme deficiencies in stock cells.

The results of the fibroblast enzyme assays were:-

Transferase assay:			
Transferase deficient	line 1	:	no detectable activity
	line 2	:	no detectable activity
	line 3	:	no detectable activity
Epimerase assay:			
Epimerase deficient	line	:	no detectable activity

The batch of foetal calf serum used throughout this period was also assayed for epimerase activity. No detectable activity was found.

Enzyme deficiencies in supplied cells:

Three externally supplied cell lines were used in experiments, these were the wild type and epimerase deficient CHO cell lines, and ED2 a human fibroblast line from the case of generalised epimerase deficiency described by Garibaldi et al(1983).

The results obtained were:

Line		Epimerase Activity (nmol UDPgal converted/mg protein/hr)
CHO LDL 14-1A clone		None detected
ED2		90
control fibroblasts	NC6	28
	NC7	42
	NC4	34

4.1.7 Cell Culture: Discussion.

As part of this project, facilities to culture cells were established, and fibroblast and Chinese hamster ovary cell cultured reproducibly and reliably. Vigilance for infection was considered to be especially important in these experiments since the lines being studied carried particular genetic characteristics and the introduction of any exogenous DNA was a possible source of error. Bacterial infection is probably the least likely to confound, since infection is generally obvious both macro- and microscopically as described in section 4.2.1, and bacteria do not generally enter the cells. In contrast, mycoplasma infection is more serious. Cultures may be affected in two ways: as an intracellular parasite mycoplasma slows down the growth of infected cells and would therefore affect several of the parameters observed in these experiments, such as rate of increase in cell number or rate of protein synthesis. Secondly, they bring new genetic material and possibly the missing enzyme into a deficient cell. Mycoplasma are notorious for persisting in cultures,

spreading between cultures in one laboratory, and being difficult to eradicate. Therefore, the frequent checks made for the presence of mycoplasma with negative results suggest that mycoplasma were not at any time present in cultures.

Infections.

Viral infections are not considered to be such a problem in fibroblast culture since significant infection is rare and usually leads to death of the culture rather than a persisting but altered culture. Interestingly, in 1971, transferase deficient fibroblasts were used in an early experiment in molecular biology (Merrill 1971). They were infected with a bacteriophage virus which carried a bacterial transferase gene. This induced the deficient fibroblasts to synthesise transferase. The infecting virus was of course formed for the experiment and was not naturally occurring. In the experience of the laboratory where this work was carried out a line tested as transferase deficient has never subsequently acquired activity spontaneously and a search of the literature produced no reports of similar cases.

Karyotypes:

Karyotypes were observed to be constant throughout the series of experiments. Changes in karyotype during culture are recognised as possible. Although such a change would not introduce new material it could affect parameters such as the rate of cell division. Passage numbers were therefore kept low to reduce the likelihood of such a change occurring, and none was observed.

Enzyme Measurements.

The enzyme measurements on all cells acquired from stocks at Southmead Hospital confirmed the expected deficiencies of transferase in lines TD 1, 2 and 3, and of epimerase in EDI. It was also important to confirm that there was no detectable epimerase activity in the batch of foetal calf serum used for these experiments. Epimerase is normally located intracellularly but foetal calf serum is a natural product containing many enzymes and proteins, and it is possible that it could contain products of lysed cells, especially erythrocytes (epimerase is present in erythrocytes). Cells in culture are able to endocytose complete proteins and enzymes from culture media (Willingham 1985) so it was necessary to establish that there was no measurable activity of epimerase in the serum used, and this was found to be the case.
The cell line ED2 was supplied by Garibaldi et al as coming from their case described in the Journal of Pedriatrics as generalised epimerase deficiency (Garibaldi 1983). However, the epimerase activity (Section 4.1.6) was found to be rather higher than the control fibroblasts. This was communicated to Dr. Garibaldi. Further assessment at Southmead Hospital showed this cell line to be transferase deficient. Garibaldi et al then published a further letter (Garibaldi 1986) saying that "We have recently become aware that a basic error exists in our paper describing a case of epimerase deficiency". He describes the results found as part of this thesis and states that a fresh sample obtained from the index case confirmed that this patient had transferase deficiency galactosaemia. He says he has no explanation for the initial results and

apologises for the erroneous information.

No detectable activity was found in the line EDI, the case detected locally. This confirms other work done on this line (Holton 1981, Gillett 1985) who were unable to find any measurable activity. However Kingsley (1986) claims that analysis of this line showed measurable activity of 9 nmol/mg/hr compared to 509 nmol/mg/hr for the single normal line measured. The paper states that the enzyme activity was determined with a thin layer chromatography assay and gives a reference to a paper entitled "Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDPGalNAc 4 - epimerase deficient mutant", which was in press. The analytical technique which allows 1.8% of activity to be discriminated from zero is therefore not described, so it is not possible to reconcile the discrepancy between this paper and the other three results. As Kingsley states in this paper, and others agree (Pillert 1983) one enzyme is responsible for epimerisation of both UDPGal and UDP N-acetyl-galactosamine (UDPGal N-Ac). It is, therefore, difficult to explain how the cell line ED1 had 1.8% of control activity for UDPGal but 11.6% of control activity for UDPGal N-Ac, if the assay is reproducible at these low levels. The paper by Kingsley also states that the clone of CHO cells had zero activity. This clone supplied by Kingsley et al was used in subsequent experiments in this work.

Asymptomatic cases of erythrocyte and leucocyte epimerase deficiency have been detected in screening programs as described in the introduction. Examination of fibroblasts from 8 such cases showed that all had detectable epimerase

activity (Gitzelmann 1978).

Similarly, activity could be stimulated by phytohaemagglutinin in 6 out of 7 deficient leucocyte lines (Mitchell 1975). These patients did not have generalised epimerase deficiency (Gitzelmann 1980), in contrast to the index case which was the origin of cell line ED1 (Holton 1981).

4.2 Experiments on the Galactose Content of Cell Culture Media.

4.2.1 Gas chromatographic analysis with mass spectrometric detection.

For the initial experiments 1mmol/L solutions of galactose and glucose in water with mannitol as an internal standard were used. The galactose solution produced two peaks at 10.3 and 12.4 minutes retention time (figure 1) and the glucose solution, two partially resolved peaks at 13.4 and 13.6 minutes retention (figure 2). Mass spectra of the larger galactose peak showed that the most suitable ions for single ion monitoring were 187, 226.1 and 331.1 atomic mass units (amu).

Galactose was added to TC 199 liquid medium to final concentrations of 0.1, 1, 10 and 100 μmol/L and derivatives prepared of these solutions. GC-MS analysis of these derivatives with selective ion monitoring at the most abundant ion (187) is shown in figures 3 to 6. Samples of TC 199 medium without any added galactose and of the batch of foetal calf serum used throughout these experiments were also analysed (figures 7 and 8).

Spectra of the 10 and 100μmol/L solutions show large peaks in the position of galactose. The 1μmol/L solution has two small peaks at the appropriate retention times. The 0.1 μmol/L solution and TC199 medium did not show any significant peaks in the position of galactose. The spectrum of foetal calf serum was more complicated and had several peaks including one coincident with the slower-running galactose peak (figure 8). Co-injection of the serum sample with a

galactose standard (figure 9) confirmed the expected position of peaks in a serum sample.

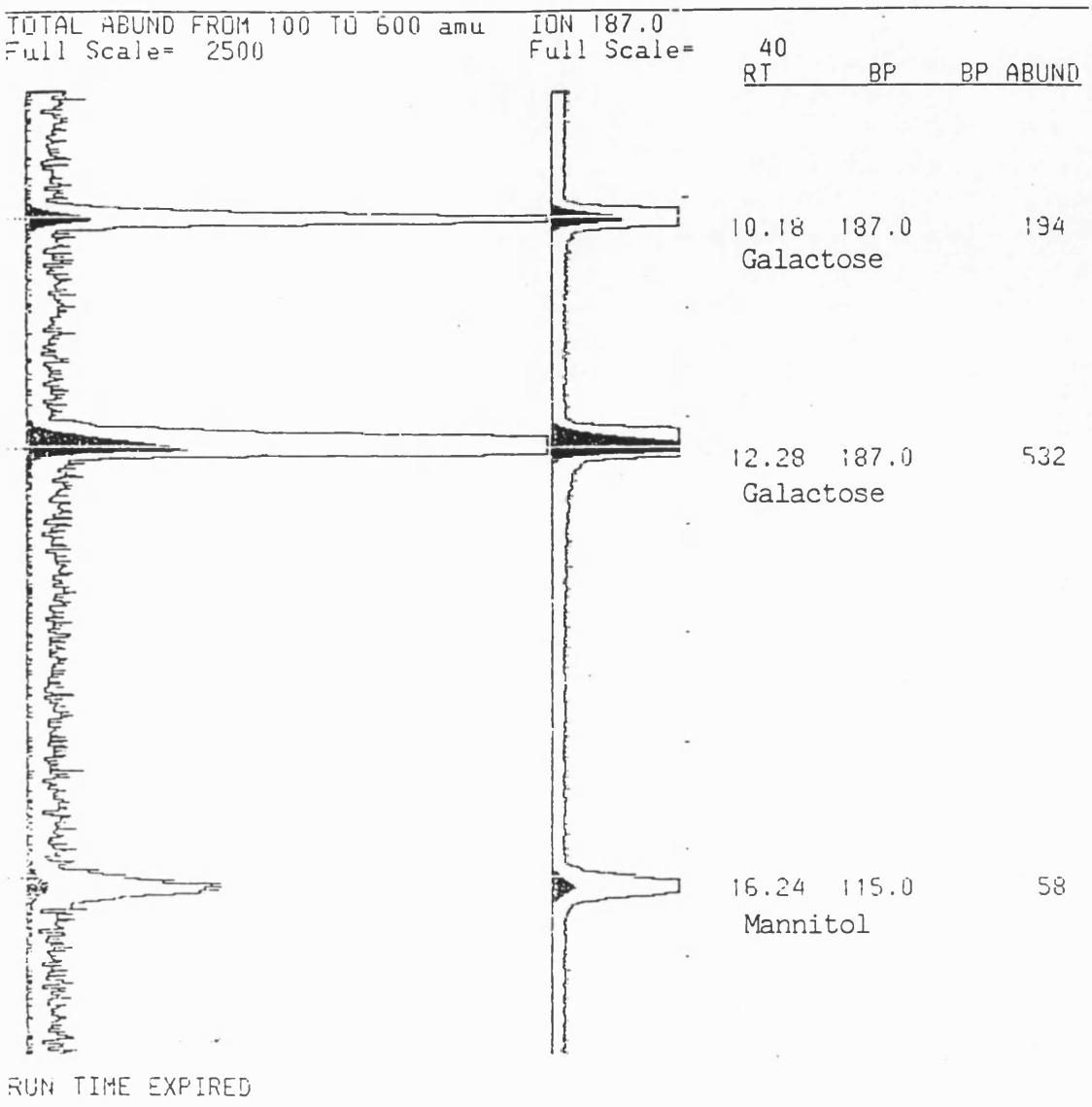


Figure 1. Gas chromatography with mass spectrometric detection of a 1 mmol/L solution of galactose using mannitol as an internal standard. The traces show total ions from 100 to 600 atomic mass units (amu) on the left hand and selective monitoring of ion 187.0 amu in the right hand trace. Retention times (RT) are shown in minutes and relative abundances (ABUND) on an arbitrary scale. Two well separated peaks are produced by galactose.

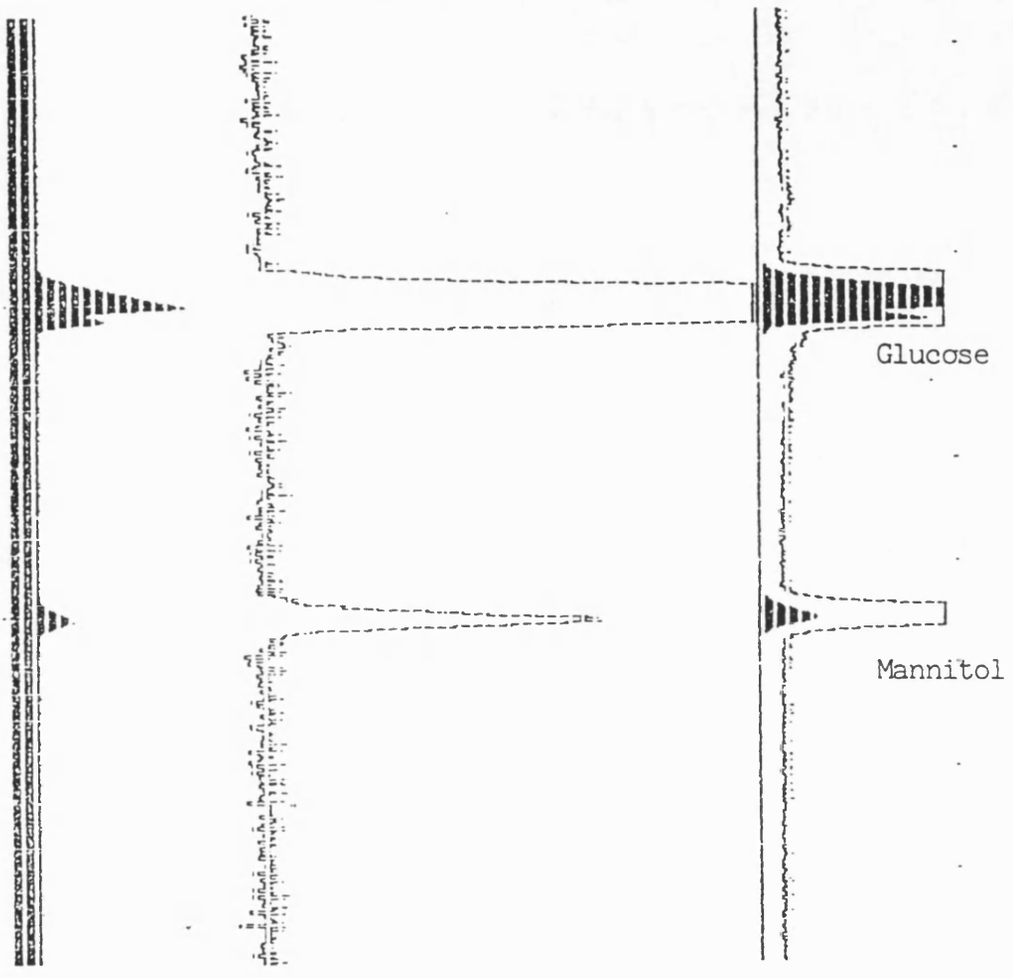


Figure 2. Gas chromatography with mass spectrometric detection of a 1 mmol/L solution of glucose using mannitol as an internal standard. Total ion monitoring of all ions from 100 to 600 amu is shown in the left hand trace, and selective ion monitoring of ion 187.0 on the right. Two partially resolved peaks are produced by glucose.

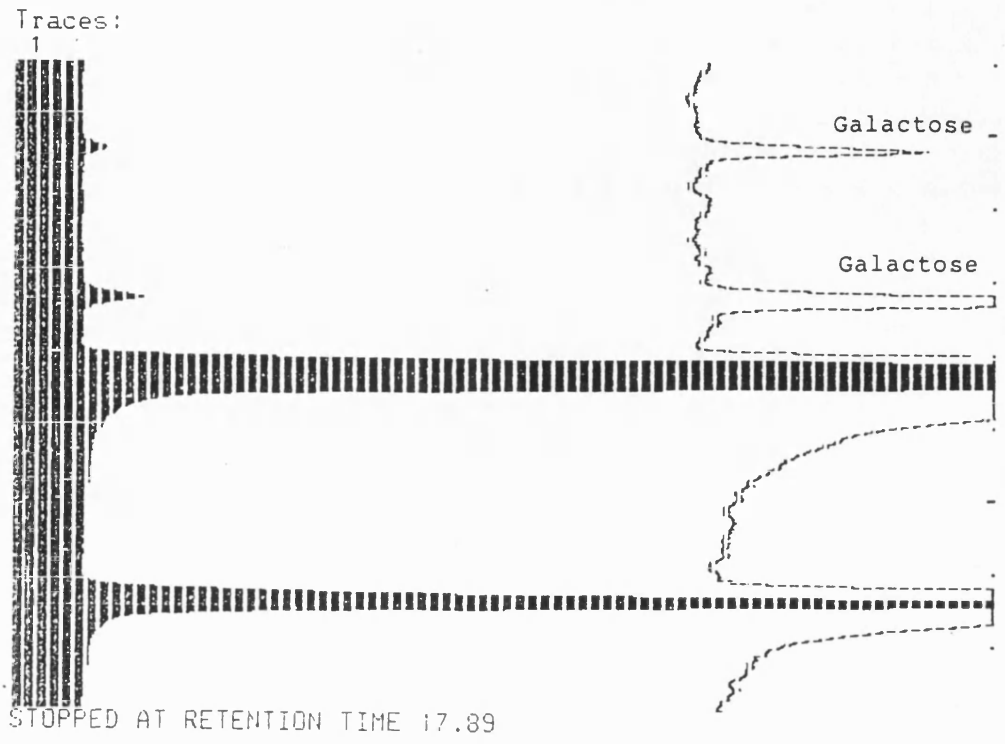


Figure 3. Gas chromatography with mass spectrometric detection using the single ion 187.0 amu of a solution of TC 199 medium with galactose added to a concentration of 100 μmol/L.

Figure 4. Gas chromatography with mass spectrometric detection using the single ion 187.0 amu of a solution of TC 199 medium with galactose added to a concentration of 10 μmol/L.

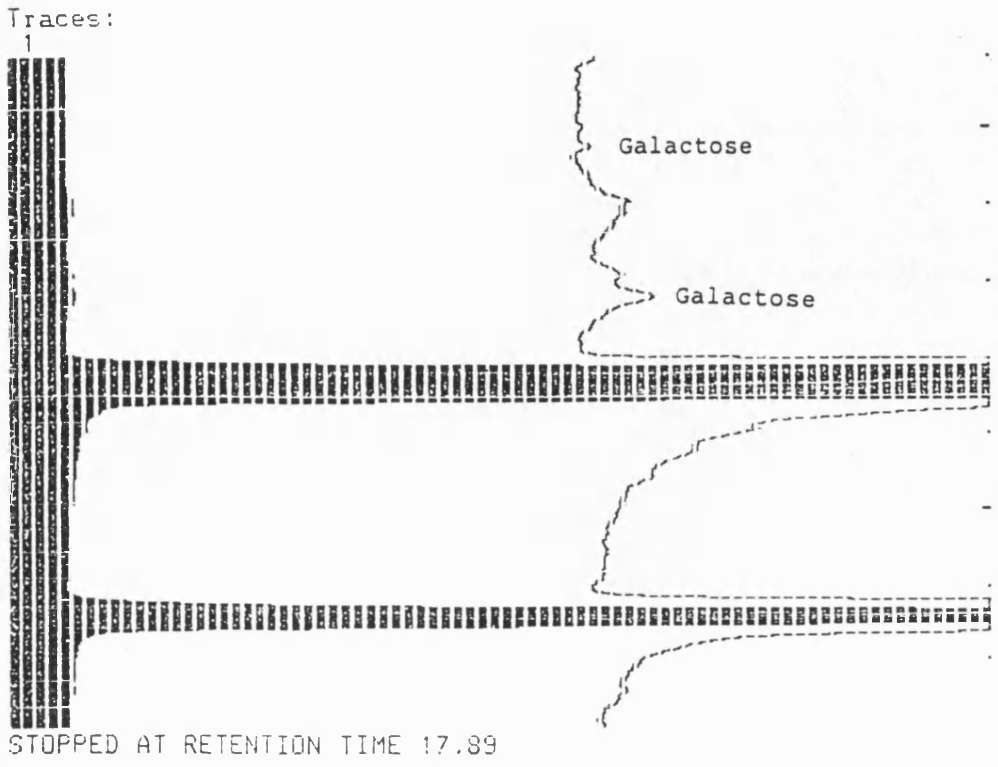


Figure 5. Gas chromatography with mass spectrometric detection using the single ion 187.0 amu of a solution of TC 199 medium with galactose added to a concentration of 1 μmol/L.

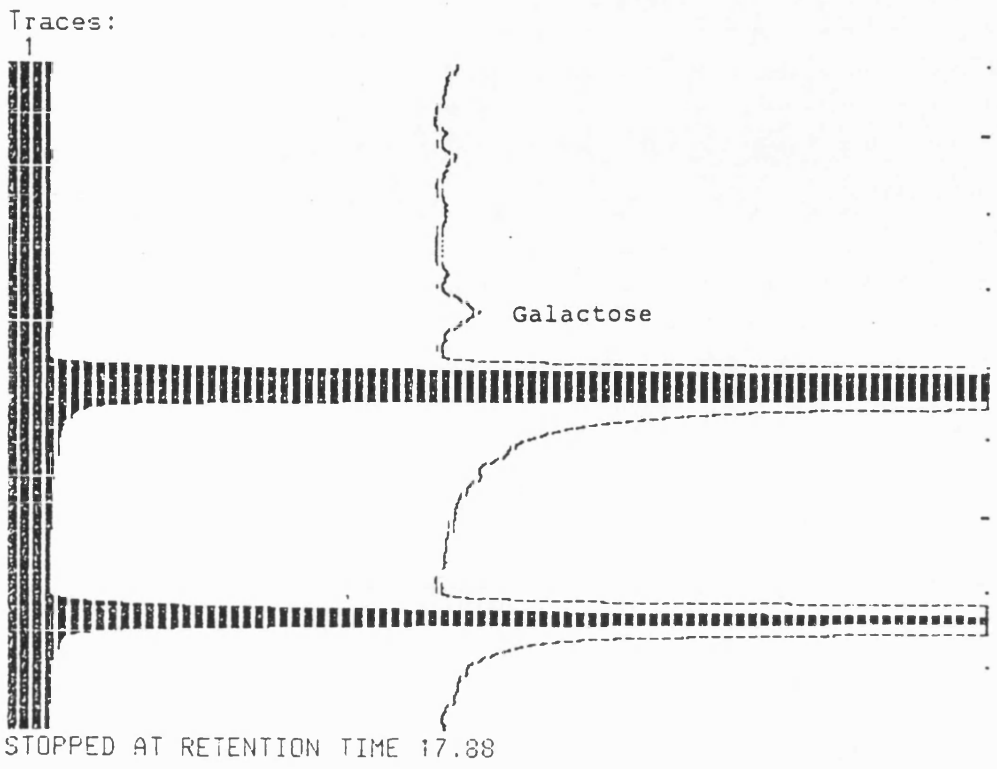


Figure 6. Gas chromatography with mass spectrometric detection using the single ion 187.0 amu of a solution of TC 199 medium with galactose added to a concentration of 0.1 μmol/L.

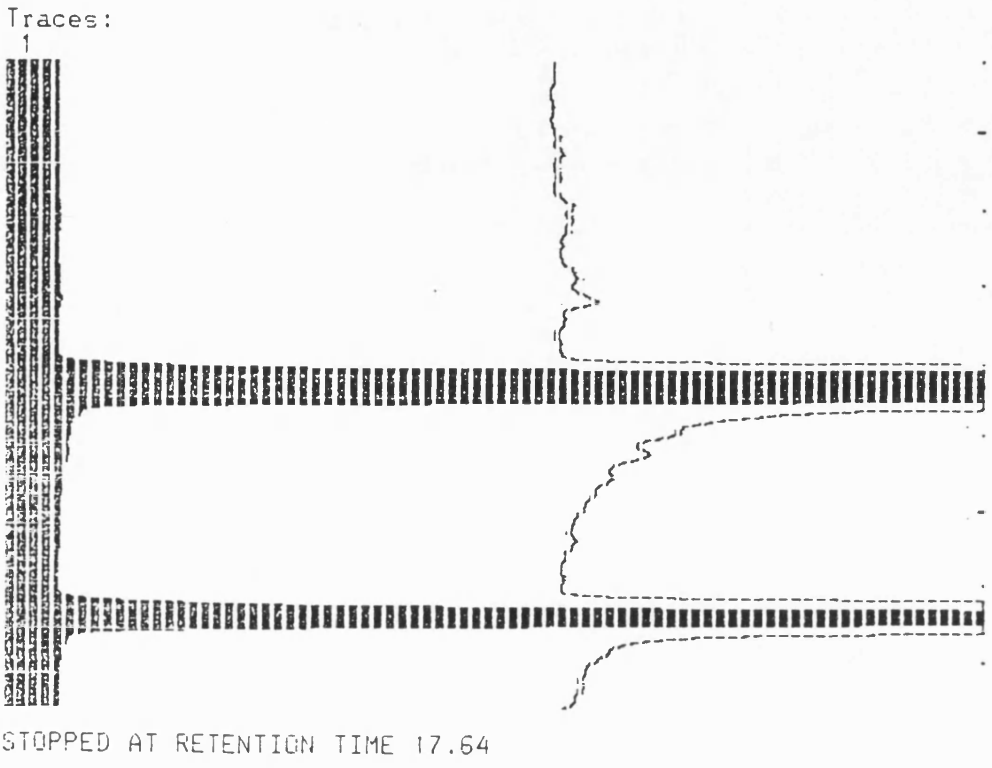


Figure 7. Gas chromatography with mass spectrometric detection using the single ion 187.0 of TC 199 medium.

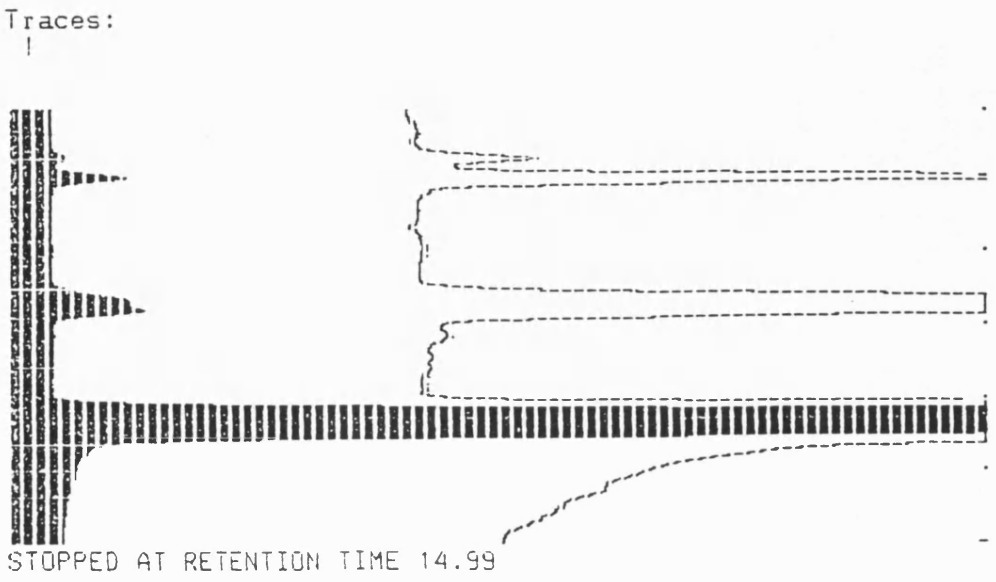


Figure 8. Gas chromatography with mass spectrometric detection using the single ion 187.0 of the batch of foetal calf serum used in these experiments.

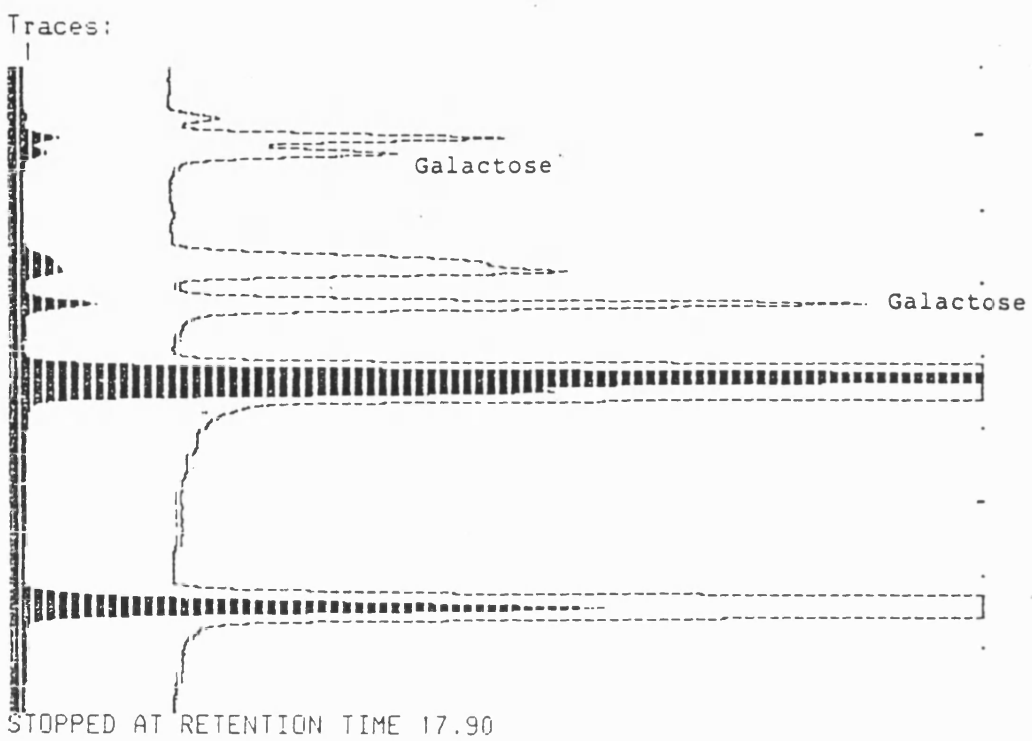


Figure 9. The sample shown in figure 8 was co-injected with a derivatised galactose solution. This confirms the expected position of the two galactose peaks.

4.2.2 Galactose containing glycoproteins in foetal calf serum.

In these experiments one ml of foetal calf serum was placed on columns of immobilised ricin lectin and eluted with 1 M NaCl solution to remove unbound protein, and 1 M NaCl with 100 mM galactose, which displaced proteins bound by their galactose residues. Eluates were collected in one ml fractions.
Elution with ten mls of NaCl solution followed by 7 mls of the galactose containing solution produced the protein levels shown in Table 2 and graphically in figure 10.
Of the 35.9 mg of protein 35.66 mg (99.3%) came out in the first four fractions. A subsiduary peak totalling 119 μg (0.33% of the total protein) was also evident, and this was eluted as soon as the galactose containing solution was placed on the columns. These results are compatable with the retention by the column of proteins in foetal calf serum which have exposed galactosyl residues. However it is also possible that the second peak represented a chromatographic retention of a proportion of the protein by some other property, and that its elution when galactose containing eluant was used was coincidental. To test this hypothesis, in the next experiment 14 mls of 1M NaCl were used (instead of 10 mls) before elution with the galactose containing solution (figure 11). Once again a small second peak was liberated immediately on changing eluants.
Next the completeness of removal of the proteins which the column was binding was investigated by running the same sample through the column three times. One ml of foetal calf serum was run through as described in the first experiment of this type. The column was regenerated and fractions 1 to 4 placed

on the column again, and eluted with 7 mls of 1 M NaCl followed by 5 mls of NaCl plus galactose. The first five fractions were placed on a regenerated column and eluted with 5 mls of NaCl and 5 mls of NaCl plus galactose. A second peak was present on each pass, but it became progressively smaller. The amounts of protein in each fraction of the second peak for the three runs are shown in figure 12, and the total amounts of protein in each of the three peaks in figure 13.

Since removal of proteins which bound to the column was clearly not complete on each run, it was possible that this amount of foetal calf serum overloaded the binding sites available. This was tested by increasing the volume of serum placed on the column and investigating whether the amount of protein bound increased proportionately. Doubling the amount of serum placed on the column resulted in an increase in the size of the second peak from 109 to 160 mg of protein.

Fraction No.	Protein level (mg/L)	Eluant
1	8300	
2	18300	
3	8400	
4	660	
5	45	1 M NaCl
6	29	
7	10	
8	5	
9	5	
10	5	
11	34	
12	46	
13	19	1 M NaCl plus
14	10	100 mmol/L
15	10	galactose
16	5	
17	5	

Table 2

Figure 10. Elution of Proteins from FITC Columns with 1 molar NaCl.

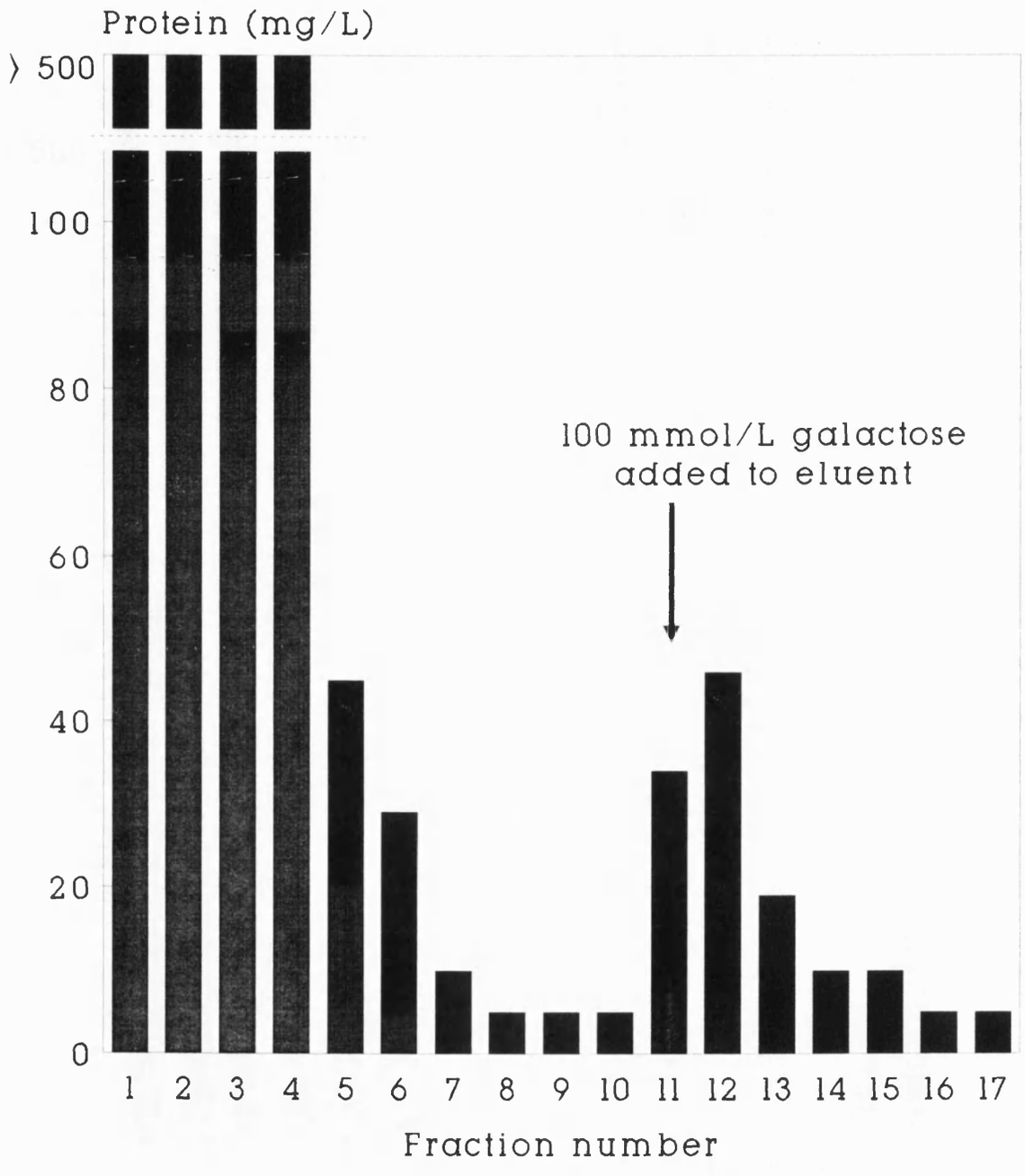


Figure 10. Affinity chromatography of proteins with exposed galactose residues. One ml of foetal calf serum was placed in a solid phase linked lectin column and 1 ml fractions collected as it was eluted, first with 1 mol/L NaCl solution to remove most proteins (fractions 1 to 10). Proteins bound to the column were displaced by eluting with 1 mol/L NaCl with 100 mmol/L galactose, producing a second peak of proteins bound by their galactose residues.

Figure 11. Elution of Proteins from FITC Columns with 1 molar NaCl.

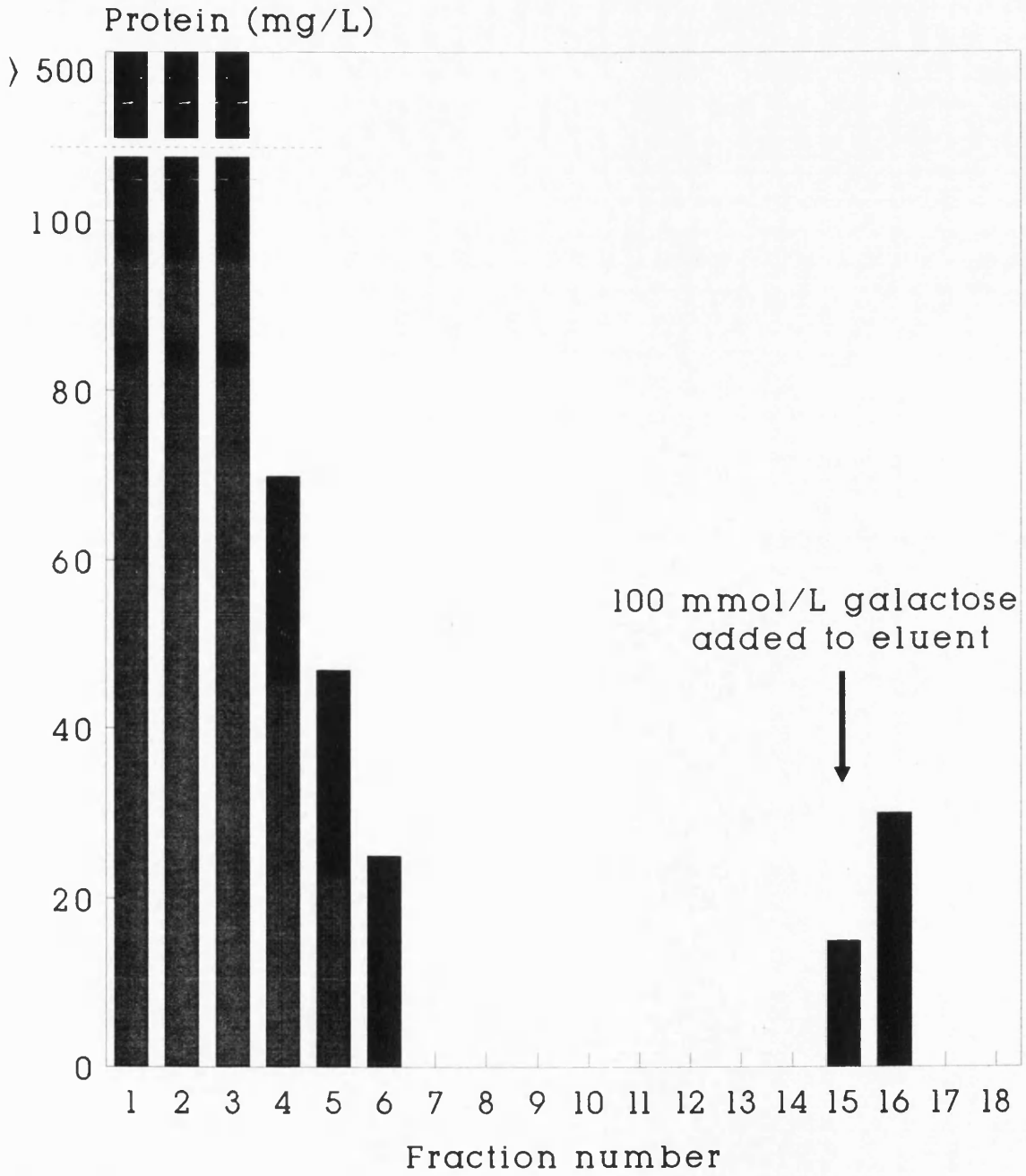


Figure 11. Affinity chromatography of proteins with exposed galactose residues. The experiment shown in figure 10 was repeated except that 14 mls of the first eluant were used. The second peak was once again not evident until a galactose containing eluant was used. This confirms an affinity effect of proteins bound by galactose residues.

Figure 12. Galactoprotein Peak- Repeated Passage of One Fraction down Column

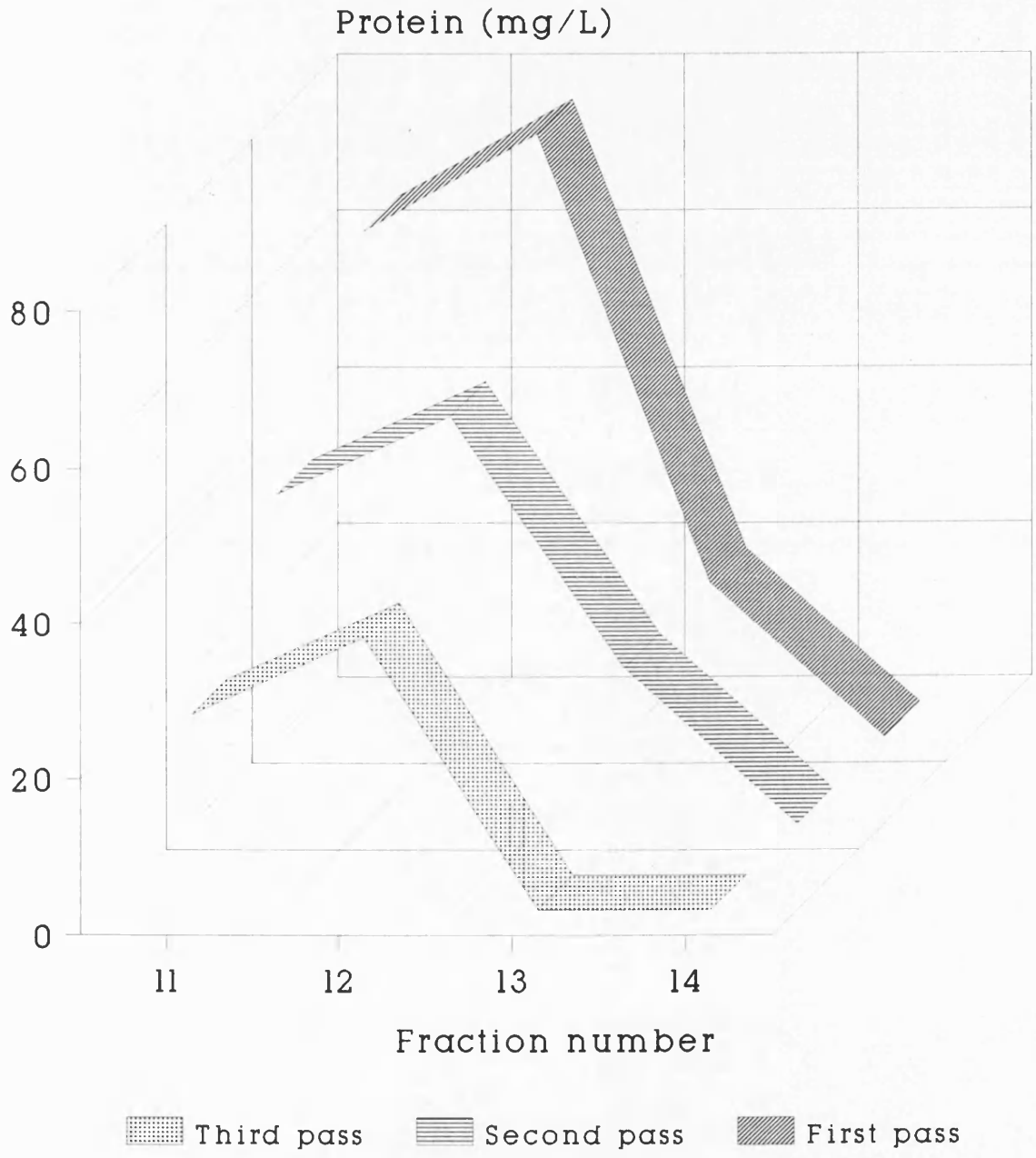


Figure 12. The completeness of removal of galactoproteins by solid phase linked lectin columns was examined by putting an aliquot of serum down a column three times and measuring the decreasing size of the galactoprotein peak with each passage. The peak was diminished with each passage, but not completely removed.

Figure 13. Total Protein on 1st, 2nd and 3rd pass

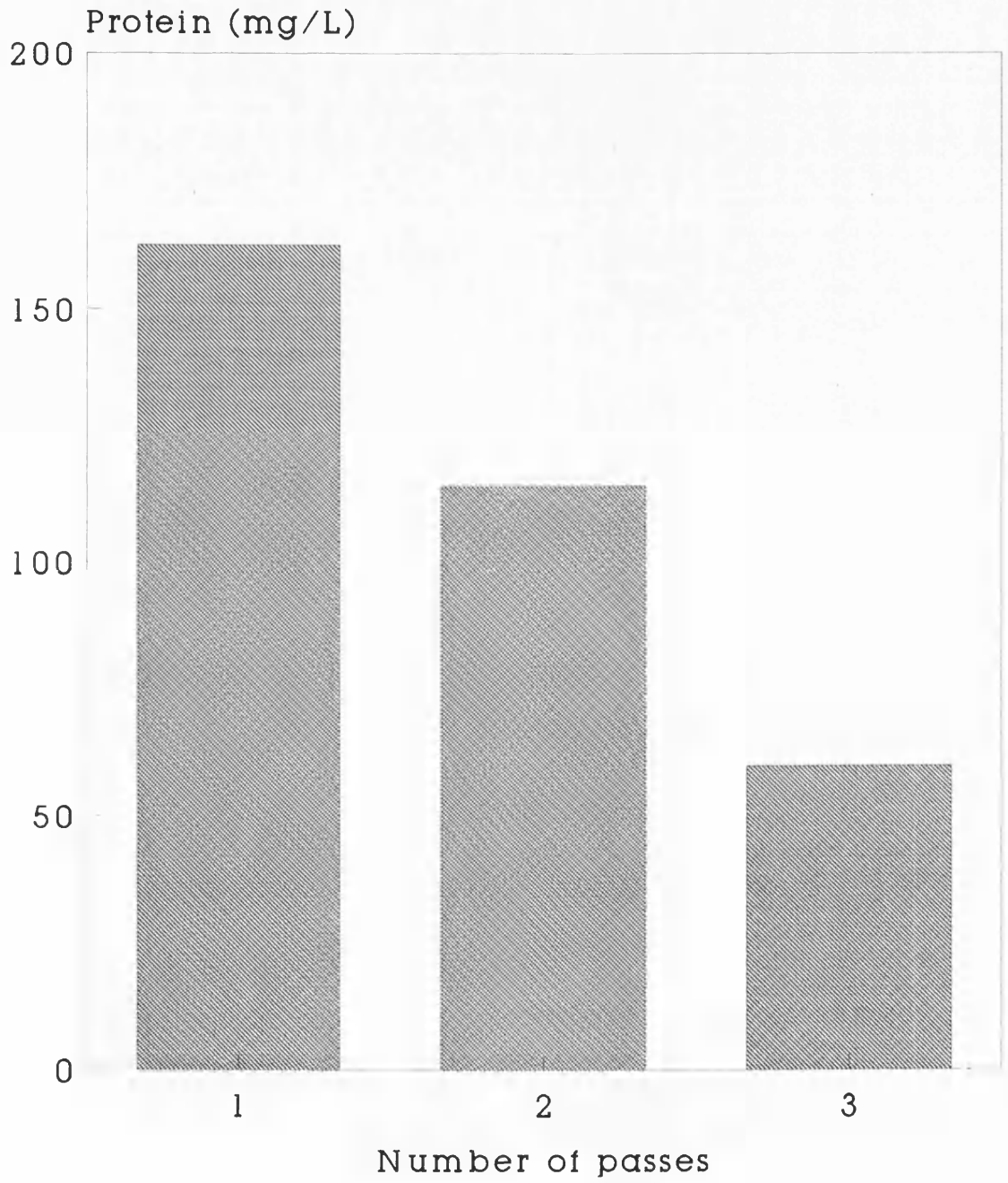


Figure 13. This shows the total amount of protein in fractions 11:14 in the three passes described in figure 12.

4.2.3 Dialysis of Serum.

This experiment investigated the conditions required for removal of uncombined galactose from foetal calf serum by dialysis. Radiolabelled galactose was added to foetal calf serum and the rate of increase of radioactivity in the dialysate measured. The results are shown in figure 14. This shows that over 90% of the dialysable radioactivity was removed in 5 hours.

4.2.4 Section 2 - Galactose Content of Culture Media: Discussion.

Much of the experimental content of this thesis concerns cell lines which are deficient in the enzymes of galactose metabolism, including two which are ostensibly unable to synthesise glactose at all. It is, therefore, essential to know as accurately as possible what the galactose content of the culture medium is, if results of experiments on the galactose content of cells are to be interpretable. Surprisingly, other workers have not considered this in the culture of epimerase deficient fibroblasts and CHO cells (Kingsley 1986) or transferase deficient fibroblasts in media described as galactose free or galactose supplemented (eg: Pourci 1990, Beratis 1987, Miller 1968).

In particular Kingsley suggests that the CHO clone used in his (and this) work is totally epimerase deficient and therefore cannot synthesise sufficient glycoprotein for structional requirements and as a consequence expresses very low levels of LDL receptors (less than 5 per cent).

Figure 14. Removal of Galactose from Serum by Dialysis

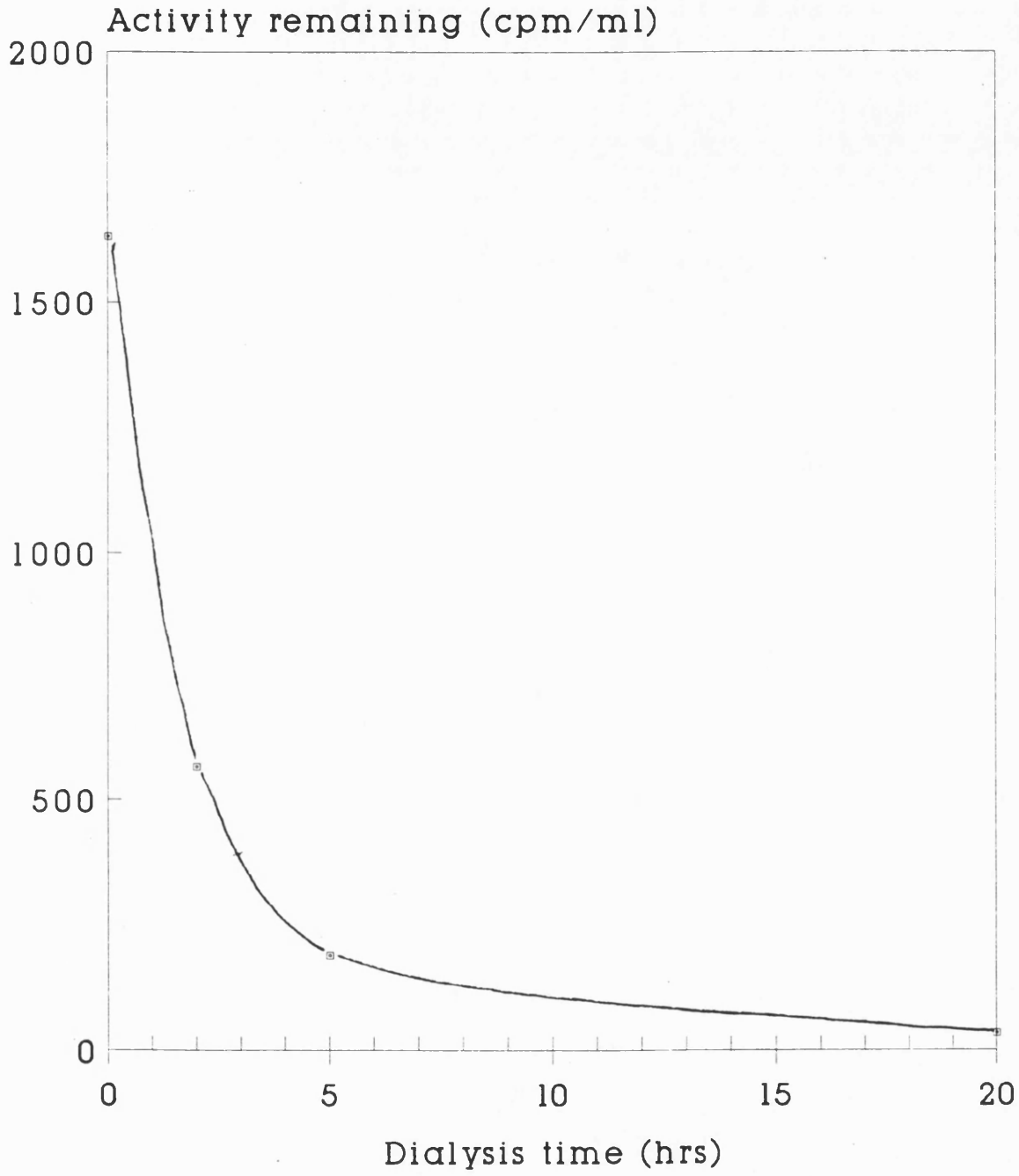
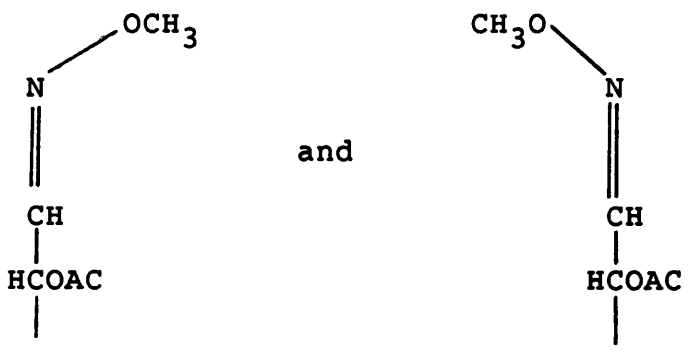


Figure 14. One microlitre of radiolabelled galactose solution was added to 40 mls of foetal calf serum and the mixture dialysed against 2 litres of normal saline at 4°C. Samples were removed from the dialysis medium at the times indicated and the activity remaining in the sac calculated.

Normal culture medium for fibroblasts consists of a buffered mixture of glucose and amino acids in water, supplemented with foetal calf serum. The serum component is the least defined and least controllable in terms of its contents. Attempts have been made to culture cells without any serum although this is more successful with transformed cell lines than normal fibroblasts (Evans 1964). Ultroser, a product marketed as a serum replacement is according to its manufacturers literature, partially derived from foetal calf serum.

The technique of gas chromatography with mass selective detection was chosen because established methods existed for the gas chromatography of monosaccharides, and these could be used with a detection system that is highly sensitive, and by monitoring spectra of peaks and choosing appropriate ions, it is also specific so that the desired analyte may be detected even under the peak of another substance.

Two peaks were produced by galactose. These are not anomers, as are often found in sugar derivitisation, since the aldehyde group was also derivitised, locking the sugar in the straight-chain form. They are syn- and anti-isomers of an imine:



The results also show that galactose can be detected by this method to low micromolar levels. A 10μmol/L solution produced significant peaks and a 1μmol/L ambiguous ones, suggesting a limit of detection of approximately 3μmol/L. No free galactose was detected in TC 199 medium or in the batch of foetal calf serum used throughout these experiments but peaks were easily discernible in spiked samples.

The next experiment in this section used columns of solid phase linked ricin lectin. There were two aims to these experiments: to demonstrate that the serum used contained proteins with exposed galactose residues, and to investigate the potential for using these columns to produce serum free of such proteins. Tsao (1981) investigated the binding of labelled cell surface glycoconjugates to columns of solid phase linked ricin lectin and showed that all labelled glycoproteins were bound to the column but glycolipids containing terminal galactosyl or N-acetylgalactosaminyl residues did not bind. The lipids probably failed to bind because they had only a single galactosyl residue by which to adhere, unlike the multiple bonding of glycoproteins.

The results of these experiments show that a proportion of the serum protein is retained on the column (fig.10), and that these proteins are displaced by the addition of galactose to the eluent. The hypothesis that galactose is displacing these proteins and that they are not simply retained chromatographically and elute co-incidentally with the change in solvent is confirmed by increasing the amount of the first eluent used: once again proteins are displaced by the addition of galactose (fig.11).

The next experiment assessed the completeness of removal (fig.12). The four galactoprotein containing fractions (10-14) were collected and put down the column once more, collected again and run through the column a third time. Each time fractions 10 to 14 gave decreasing amounts of glycoprotein as will be seen from figure 13 which shows the glycoprotein in fractions 10 to 14 for each run. This shows that after three passages, fractions 10 to 14 still produced a solution containing 60mg/L of glycoprotein, compared to 162mg/L on the first passage, although it was also demonstrated that the binding sites on the column were not fully saturated by increasing the amount of serum used. It is, therefore, clear that while the columns significantly reduce the amount of glycoprotein with exposed galactosyl residues, to produce a serum that gives no measurable second peak at all would take a very large number of passages. The experiments on the dialysis of labelled galactose showed that free galactose could be dialysed out of serum under the conditions described, and would be equilibrated in about 12hrs (fig.14). As a result of this, dialysed serum was subsequently produced by allowing equilibration overnight. Three periods of dialysis using the volumes described will lower the galactose concentration by a factor of 10^5. The GC-MS analysis of media showed a galactose concentration significantly below 10μmol/L, therefore the concentration of free galactose in dialysed serum would be below 100picomoles/L.

4.3 Experiments on cell growth rates in normal media.

These experiments investigated the rate at which cells with enzyme deficiencies grew in comparison with control fibroblasts in normal culture medium, that is with no added galactose. Four measures of cell growth were assessed independently. These were the increase in cell number, the rate of radiolabelled leucine incorporation which reflects the rate of protein synthesis in the cell, and the rates of radiolabelled uridine and thymidine uptake showing respectively the rates of RNA and DNA synthesis by the cells.

4.3.1 Cell Numbers.

These were assessed at four points over 7 days. Measurements were made on three control fibroblast lines, three transferase deficient lines and one epimerase deficient line, and on the epimerase deficient and control CHO cell lines. For all cell lines, each measurement was made in quadruplicate and the results are plotted in figure 15 for fibroblasts and figure 16 for CHO cells. Figure 15 shows that normal and transferase deficient cells increase in number at similar rates, but that the epimerase deficient line increases at a significantly slower rate. In contrast, Figure 16 shows that epimerase deficient CHO cells increase at very similar rates to control CHO cells.

4.3.2 Protein Synthesis Rates.

These experiments were carried out in two groups. In the first, two normal controls, two transferase deficient lines and one epimerase deficient fibroblast line were used, and

Figure 15. Growth of Fibroblasts in Galactose-Free Medium.

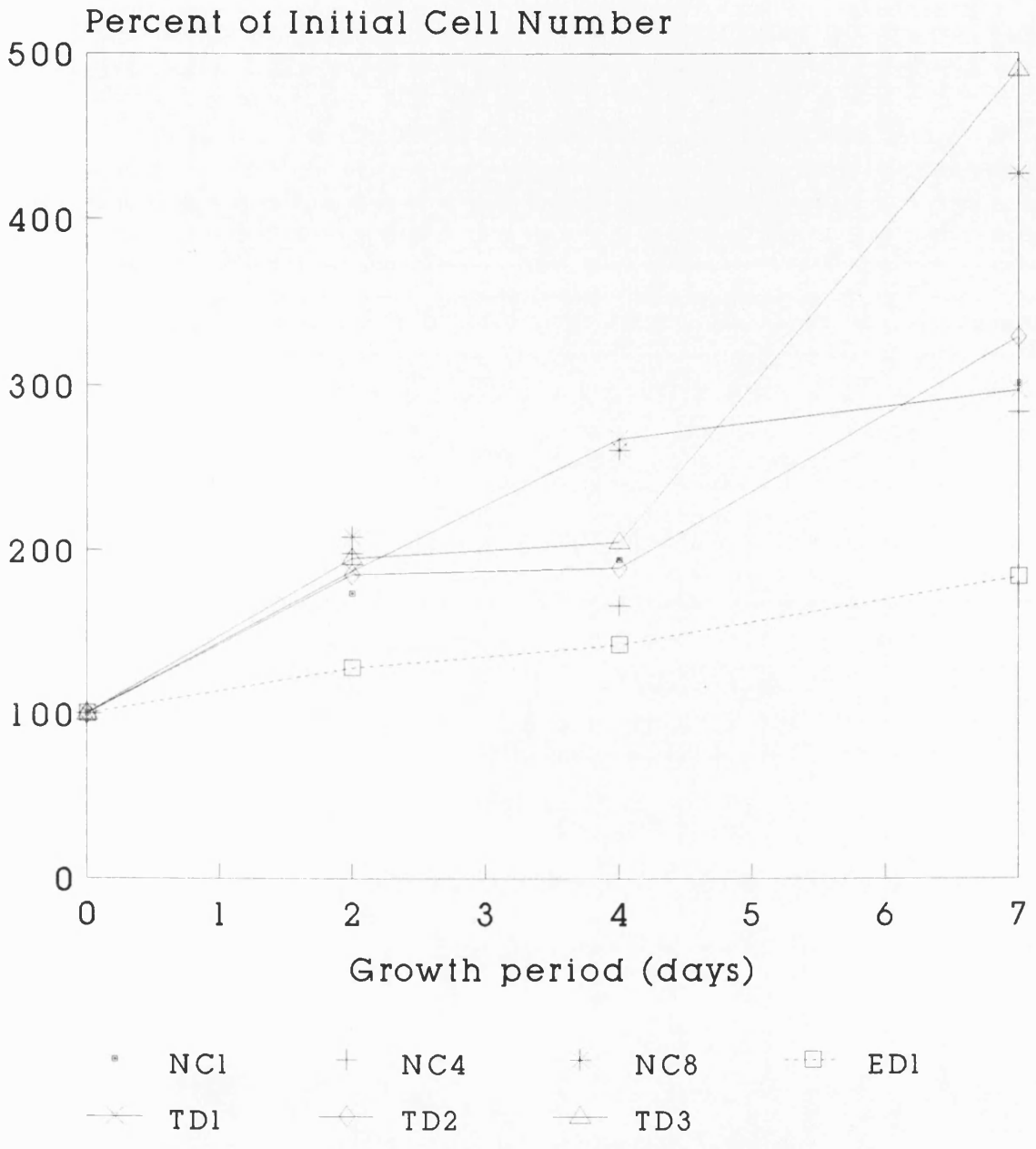


Figure 15. The growth of three normal control (NC) lines, three transferase deficient (TD) and one epimerase deficient line (ED1) in normal medium was investigated. A suspension of each cell line was placed in 16 4 cm^2 wells of a multiwell plate and left for 24hrs to allow adhesion. Four wells were then trypsinised and the cells counted (time 0). Further quadruplet counts were made at 2, 4 and 7 days.

Figure 16. Growth of CHO Cells in Galactose-Free Medium.

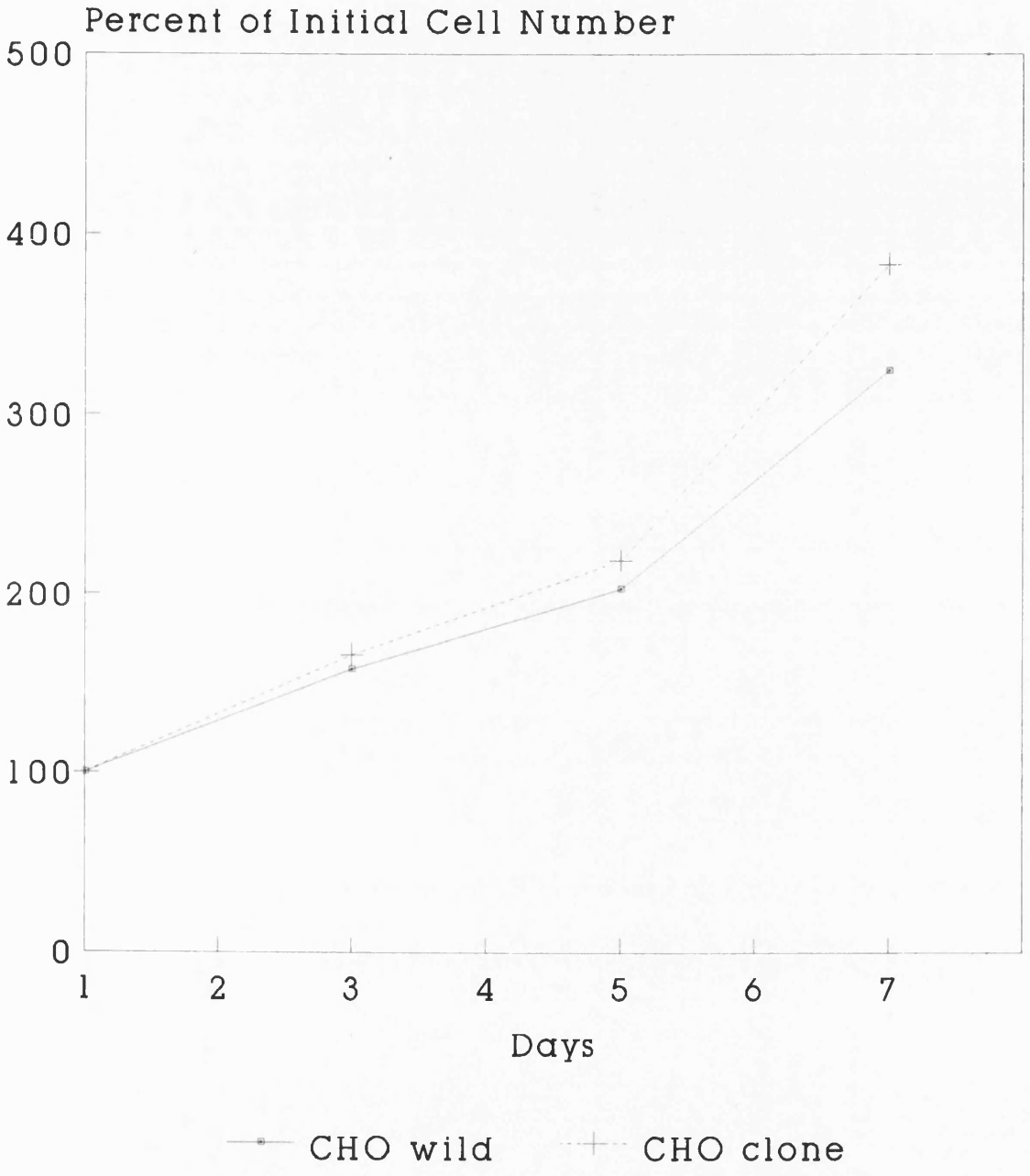


Figure 16. The growth of CHO wild type and epimerase deficient clone cells in normal medium was examined. The conditions were the same as in figure 15.

these results are shown in figure 17. In the second, three transferase deficient lines, one normal control and one epimerase deficient line, were used (fig 18). Measurements were made in triplicate, the highest, lowest and mean are shown.

Results were calculated by subtracting a blank value (from wells which had radiolabelled medium added at the end of the experiment) from the counts observed in 300 seconds for the test wells and dividing this by the amount of protein present per well. Results were normalised to the epimerase deficient line equaling 100 in order to show by what extent other lines synthesised protein at faster or slower rates. It is evident that the transferase and epimerase deficient lines do not incorporate leucine at a significantly different rate from control cell lines.

4.3.3 Uridine Incorporation Rate.

This experiment was carried out on four normal control fibroblast lines, three transferase deficient and one epimerase deficient line, and the wild type and epimerase deficient CHO cell lines. Four observations were made on each cell line : these are shown, together with the mean and standard deviation in Figure 19. The maximum percentage of the label incorporated in any one well in the fibroblast lines was 3.9% by the transferase deficient line TD2, and 6.2% in the epimerase deficient CHO cell line. The results show that the epimerase deficient fibroblast line incorporated uridine at a comparable rate to control fibroblasts, but transferase deficient lines had a significantly higher rate. There was

Figure 17. Leucine Incorporation Rates.

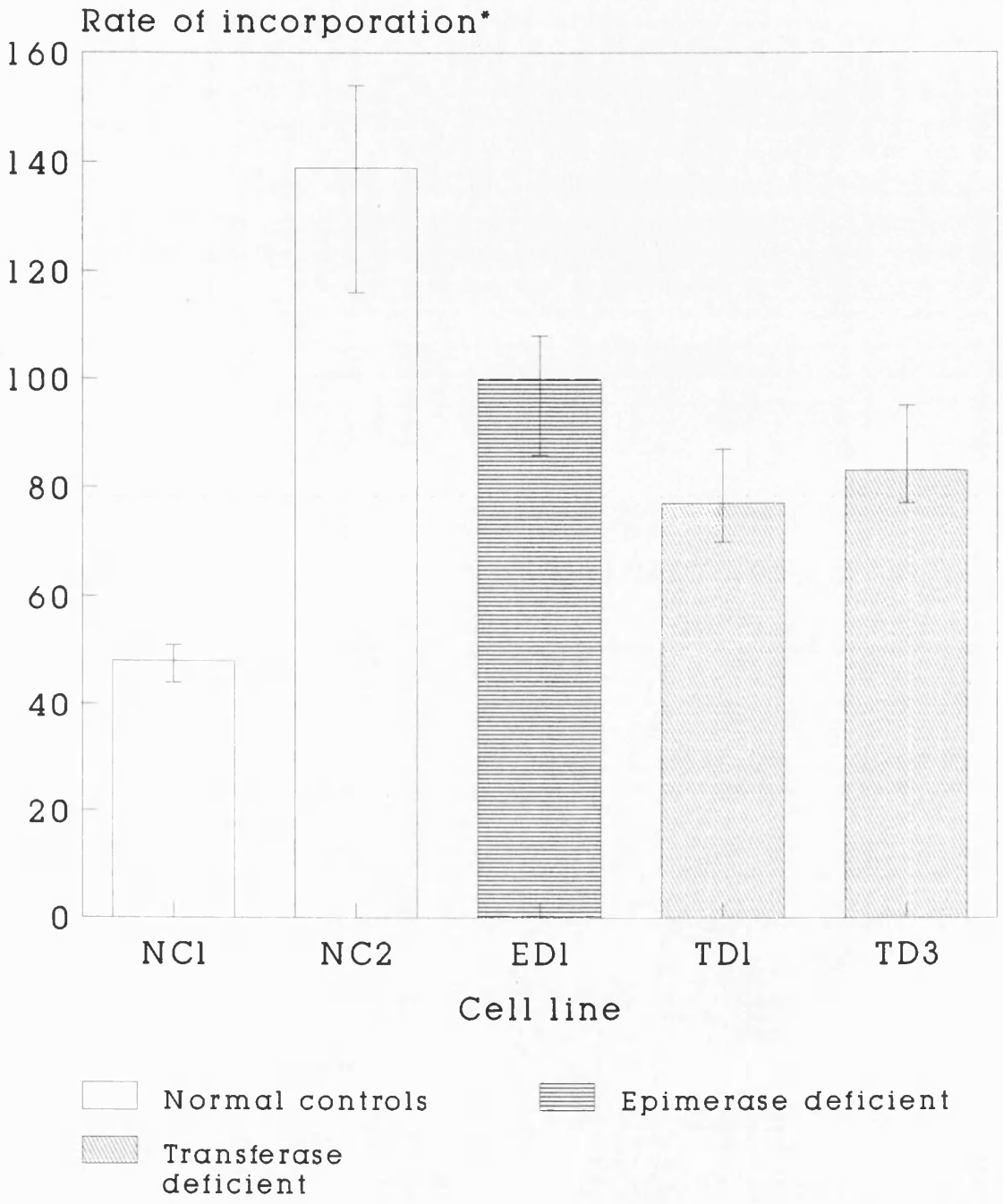
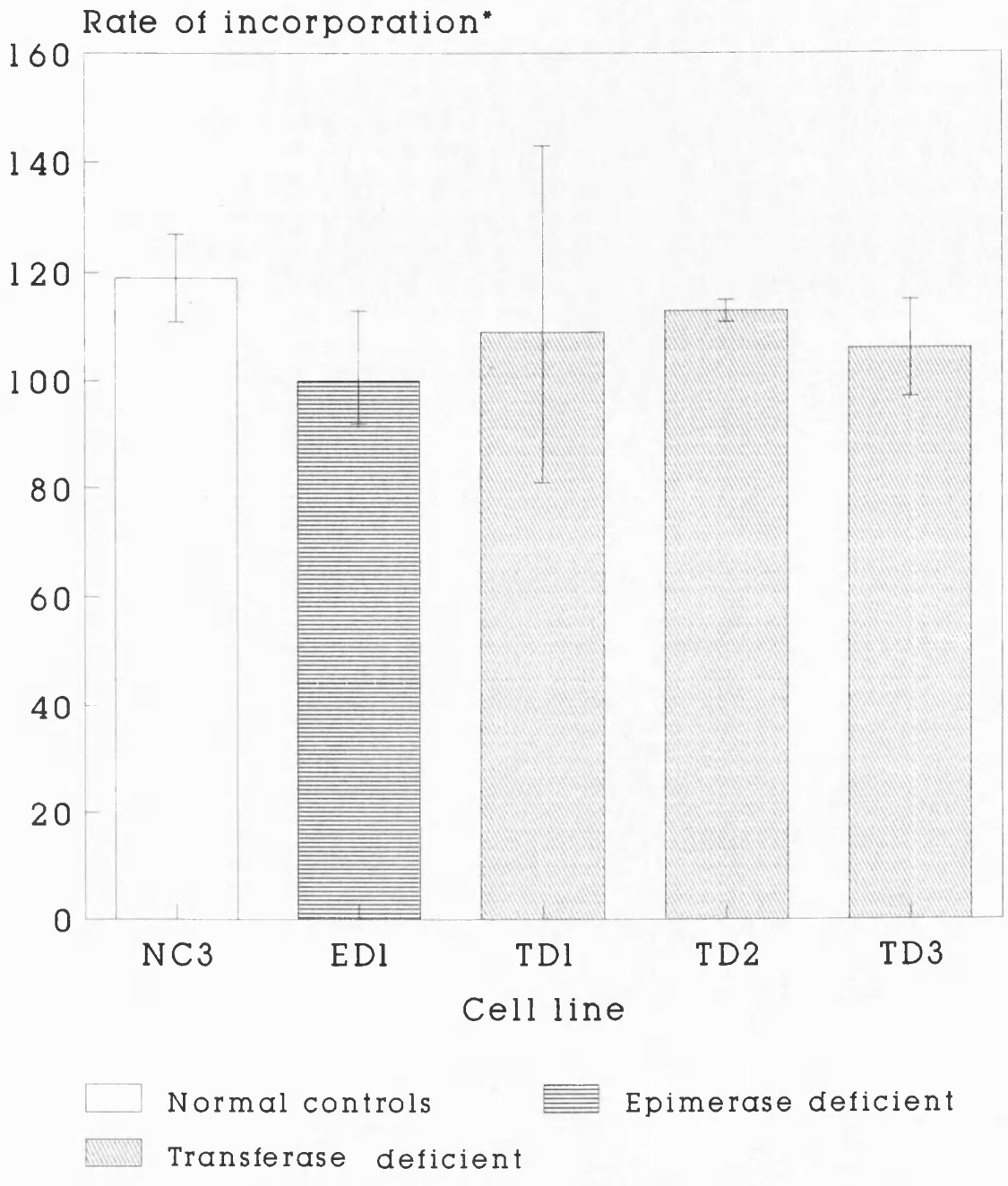


Figure 17. Normal control, epimerase deficient and transferase deficients cells were grown in multiwell plates. The medium was removed from test wells and replaced with medium containing 1 µCi/ml of ^{3}H leucine. After 1hr medium in blank wells was removed, replaced with the radiolabelled medium then all wells washed. The radioactivity incorporated and protein per well were then estimated by β-counting as described in the text. The incorporation per unit protein was expressed as a percentage of that for cell line ED1.

Figure 18. Leucine Incorporation Rates.



*Results expressed as % of ED1 activity.

Figure 18. Leucine incorporation rates were examined for further cell lines as described in figure 17.

Figure 19. Uridine Incorporation Rates

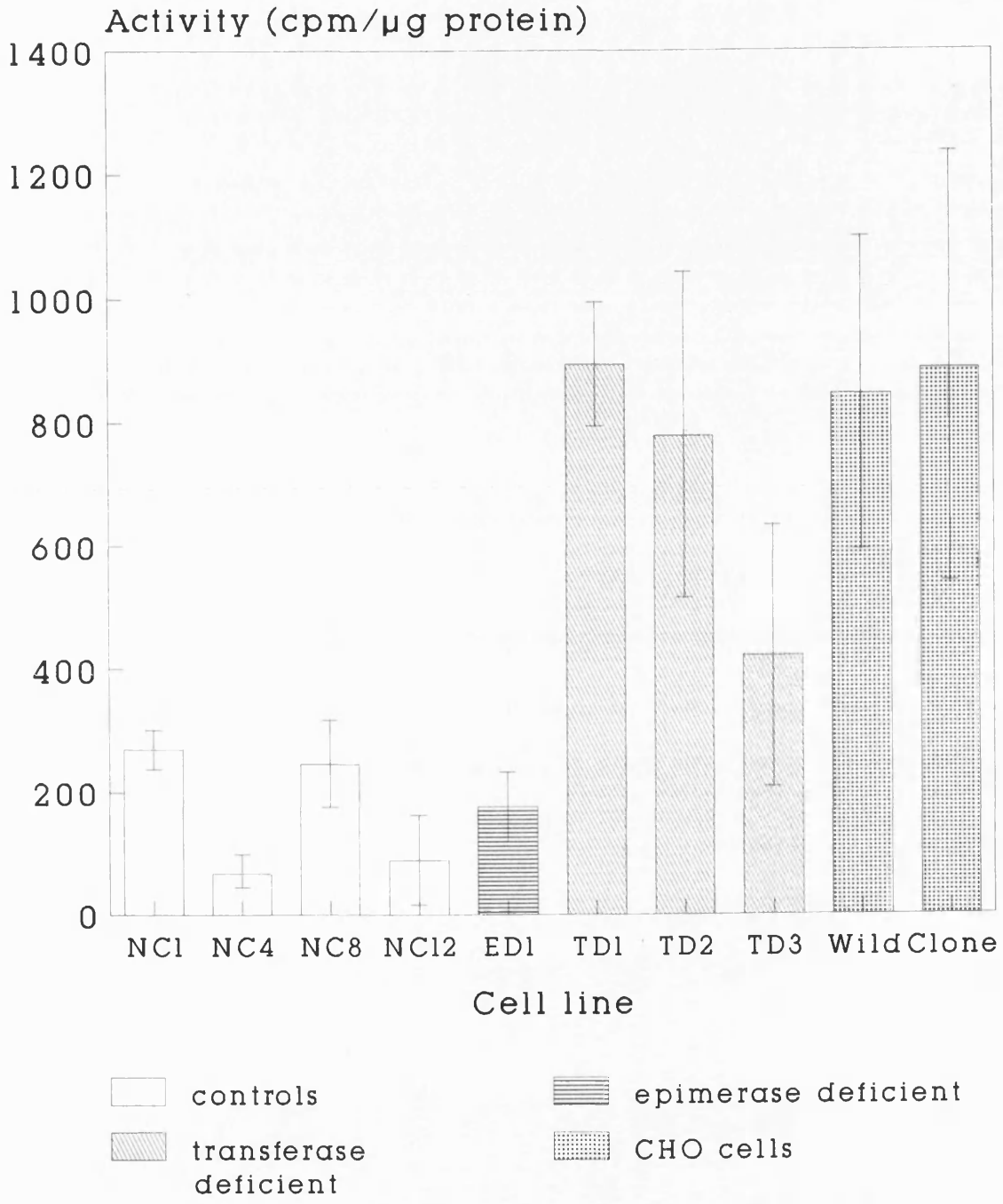


Figure 19. Uridine uptake was assessed by a similar method to that described in figure 17, except that medium containing 1µCi/ml of ^{3}H uridine was used. Results are expressed as counts minute per µg of proteins.

no significant difference between the two CHO lines.

4.3.4 Thymidine incorporation rate.

This was assessed in the same cell lines as were used in experiment 4.3.3 : Uridine Incorporation Rate. The experiment was carried out and results calculated the same way as in the previous experiment, except that radiolabelled thymidine replaced uridine. The results are shown in figure 20. As with experiment 4.3.3 the epimerase deficient fibroblasts are not significantly different from control fibroblasts but transferase deficient lines had a higher average incorporation rate than controls. Again, there was no significant difference between epimerase deficient and wild type CHO cells.

4.3.5 Section 3: Cell growth in Normal Media - Discussion.

These experiments were carried out in media to which no galactose had been added: that is medium which in Section 2 was shown to contain less than one nanomole per litre of free galactose but which did contain glycoprotein with exposed galactosyl residues. It is important to note however that this does not mean that the metabolism in transferase deficient cell lines will be normal. In vivo galactosaemic subjects will have significant levels of gal-1-P in erythrocytes despite a galactose-free diet and in vitro galactosaemic erythrocytes accumulate gal-1-P when incubated with glucose (Gitzelmann 1980), and galactosaemic fibroblasts do likewise (Pourci 1990).

The simplest and most important observation from the

Figure 20. Thymidine Incorporation Rates

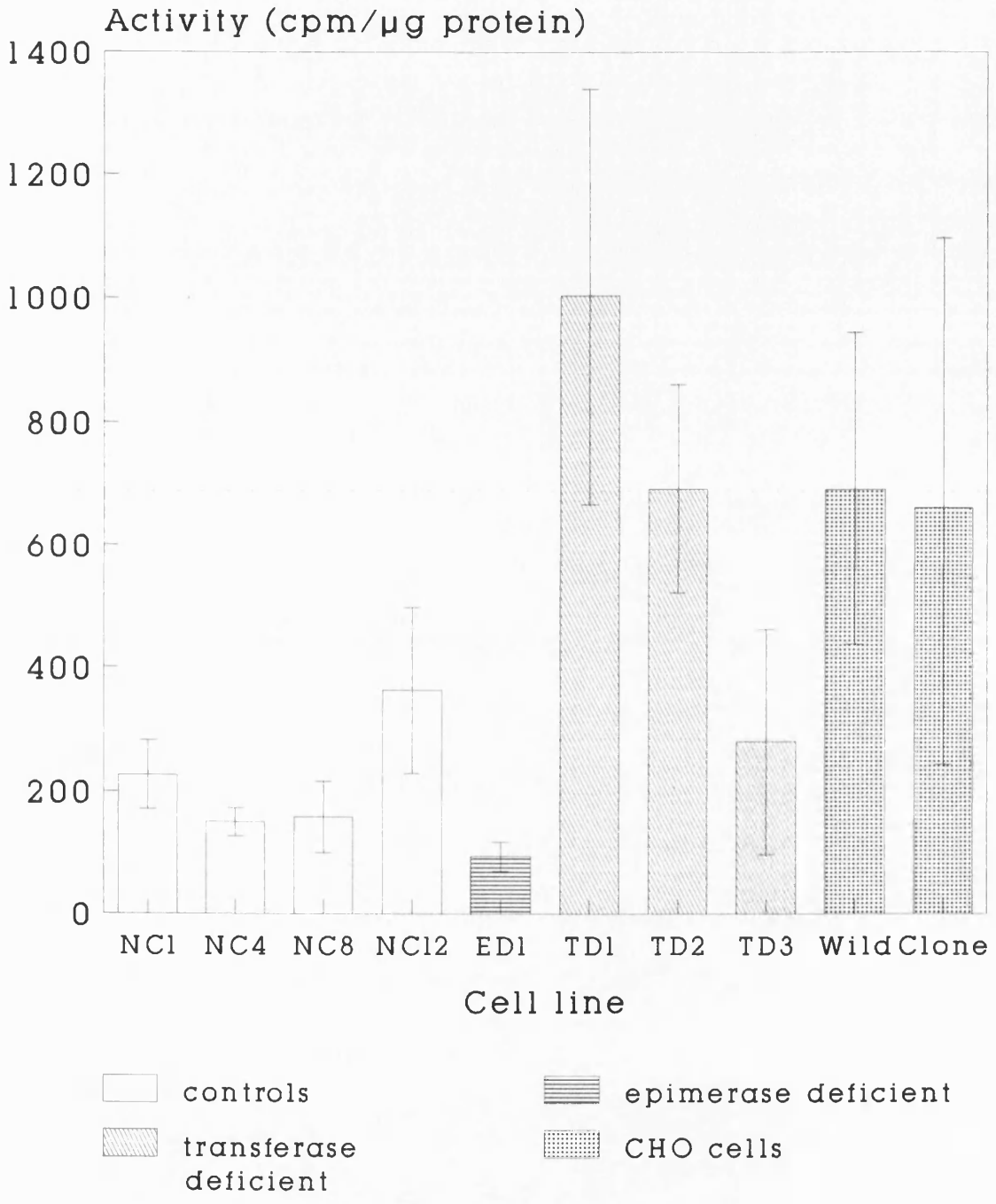


Figure 20. Thymidine uptake was assessed as in figure 17 except that medium containing 1µCi/ml of ^{3}H thymidine was used. Results are expressed as counts per minute per µg of protein.

experiments on cell number is that both transferase and epimerase deficient cell lines increased in number when cultured with glucose as their carbohydrate source. Transferase deficient fibroblasts have been shown to grow in culture previously (Krooth 1960, Pourci 1990) but have not been quantitatively compared with control fibroblasts. The results in Figure 15 show no difference between transferase deficient fibroblasts and controls. This suggests that any abnormalities in gal-1-P levels or nucleotide metabolism are insufficient to compromise the rate at which the cells grow over this timescale.

Epimerase deficient fibroblasts steadily increased in number, but at a slower rate than transferase deficient or control fibroblasts. The observation that they grow in culture is very important. In his description of partial epimerase deficiency (ie that which is detected by screening and which produces no clinical symptoms) Gitzelmann (1980) considers that complete deficiency would be incompatible with life. Similarly epimerase deficient CHO cells also grow at about the same rate as unaffected controls. This is noteworthy since this cell line is reported as being so galactose depleted that it cannot synthesise the structural protein for LDL receptors (Kingsley 1986). There are three possible explanations for the observation that epimerase deficient cells can grow in unsupplemented medium. These are that:

1. Some residual enzyme activity exists which is sufficient to supply the needs of the cell for structural carbohydrate.

2. Another sugar is used in place of galactose
3. The cells are able to abstract from their medium either galactose or an enzyme to synthesise it.

These possibilities will be discussed in the light of subsequent and previous experimental observations in the Conclusions section of this Thesis.

It is however, clear that, by whatever method or methods, the epimerase deficient CHO cells are able to overcome any constraints on their growth that their deficiency exerts. It must be concluded that they are not, therefore, a suitable model for the study of epimerase deficiency in humans.

In the experiments on protein synthesis rates, no significant difference was found between lines. However, in the previous experiment, the ED1 cell line was shown to increase in number more slowly than the other lines. This suggests that either the ED1 cells contain more protein that others, or that the protein turnover per cell is higher, for instance if carbohydrate formation was out of step with protein synthesis, leading to an increased turnover of defective glycoproteins. Xu et al (1989a) have suggested that in vivo complications of galactosaemia such a premature ovarian failure are due to disturbed glycoprotein synthesis.

The experiments on uridine and thymidine uptake gave some very significant results. Uptake of both uridine and thymidine was significantly higher in transferase deficient fibroblasts than in controls or epimerase deficient fibroblasts. It is also clear that the relative rates of incorporation (TD1 > TD2 >> TD3) is reproducibly common to both uridine and thymidine.

It is becoming evident that the biochemical abnormalities of

transferase deficiency are related to disturbance of purine and pyrimidine metabolism. Forster (1975a) found that an oral galactose load caused an increase in serum urate in galactosaemics but none in controls, and proposed that depletion of nucleotides is the cause of galactosaemic liver damage. He also showed (1975a) that incubation of normal human leucocytes with galactose caused a depression of UTP, UDP and UDP glu and accumulation of UDP gal, and that these effects were absent from leucocytes from galactosaemic patients. Pourci (1990) demonstrated that transferase deficient cells grown in galactose containing medium died, but if inosine was added to the medium, they grew normally, despite high levels of gal-1-P. Ng (1989) found that levels of UDP gal were decreased in vivo in galactosaemic red blood cells and in vitro in transferase deficient fibroblasts. The results of increased uptake described in Sections 4.3.3 and 4.3.4 could be attributed to greater rates of cell division (hence increased DNA and RNA synthesis) with an increased rate of cell death, since the proliferation of cells observed in Section 4.3.1 was the same as that of controls. Tedesco (1979) found that when transferase deficient cells were grown with galactose as their carbohydrate source their uptake of uridine was not significantly different from controls. These data are compatable with the observations that transferase deficient cells grown with glucose as their carbohydrate source take up more uridine, but the addition of galactose suppresses growth.

4.4 Cell Growth in Media Supplemented with Galactose

These experiments assessed the growth of cells in media supplemented with galactose at concentrations up to 5 mmol/L. Parameters assessed were change in cell number, rate of incorporation of radiolabelled leucine and amount of protein per well in multiwell plates, and morphology which was recorded photographically.

4.4.1 Cell numbers

Cells were grown in medium supplemented with 5 mmol/L galactose. Four wells were harvested for counting at times 0, 3, 6 and 12 days for each cell line under investigation. Three transferase deficient, one epimerase deficient and three control fibroblast lines were used, along with the CHO wild type and epimerase deficient clone. The mean and SD of each set of four observations are shown in Table 3 and the results plotted in figures 21 (control fibroblasts), 22 (transferase and epimerase deficient fibroblasts) and 23 (CHO cells). Three types of growth curve are evident in the results of this experiment. Control fibroblasts and control CHO cells increase steadily in number throughout period of observation. Transferase deficient lines in contrast all show a drop in number between the 6th and 12th days. Lastly both epimerase deficient lines (fibroblast and CHO cells) show a continuous decline. By day 6, the CHO clone cells appeared on microscope examination to be reduced in size and had rounded up. By day 12 no CHO clone cells were alive.

Cell Line	Day 0	Day 3	Day 6	Day 12
NC1	100 (11)	109 (13)	131 (14)	-
NC4	100 (14)	130 (20)	168 (21)	202 (17)
NC8	100 (3)	153 (22)	203 (40)	243 (37)
TD1	100 (7)	159 (15)	241 (28)	165 (14)
TD2	100 (5)	223 (30)	345 (120)	300 (55)
TD3	100 (13)	99 (15)	130 (10)	116 (27)
ED1	100 (21)	78 (9)	70 (12)	55 (11)
CHO wild	100 (18)	184 (24)	193 (13)	329 (92)
CHO clone	100 (22)	75 (7)	19 (16)	0

Table 3 - Change in cell numbers in galactose-enriched medium. Numbers normalised to time 0 = 100. Mean with standard deviation in brackets shown.

Figure 21. Growth of Control Fibroblasts in Galactose Enriched Medium

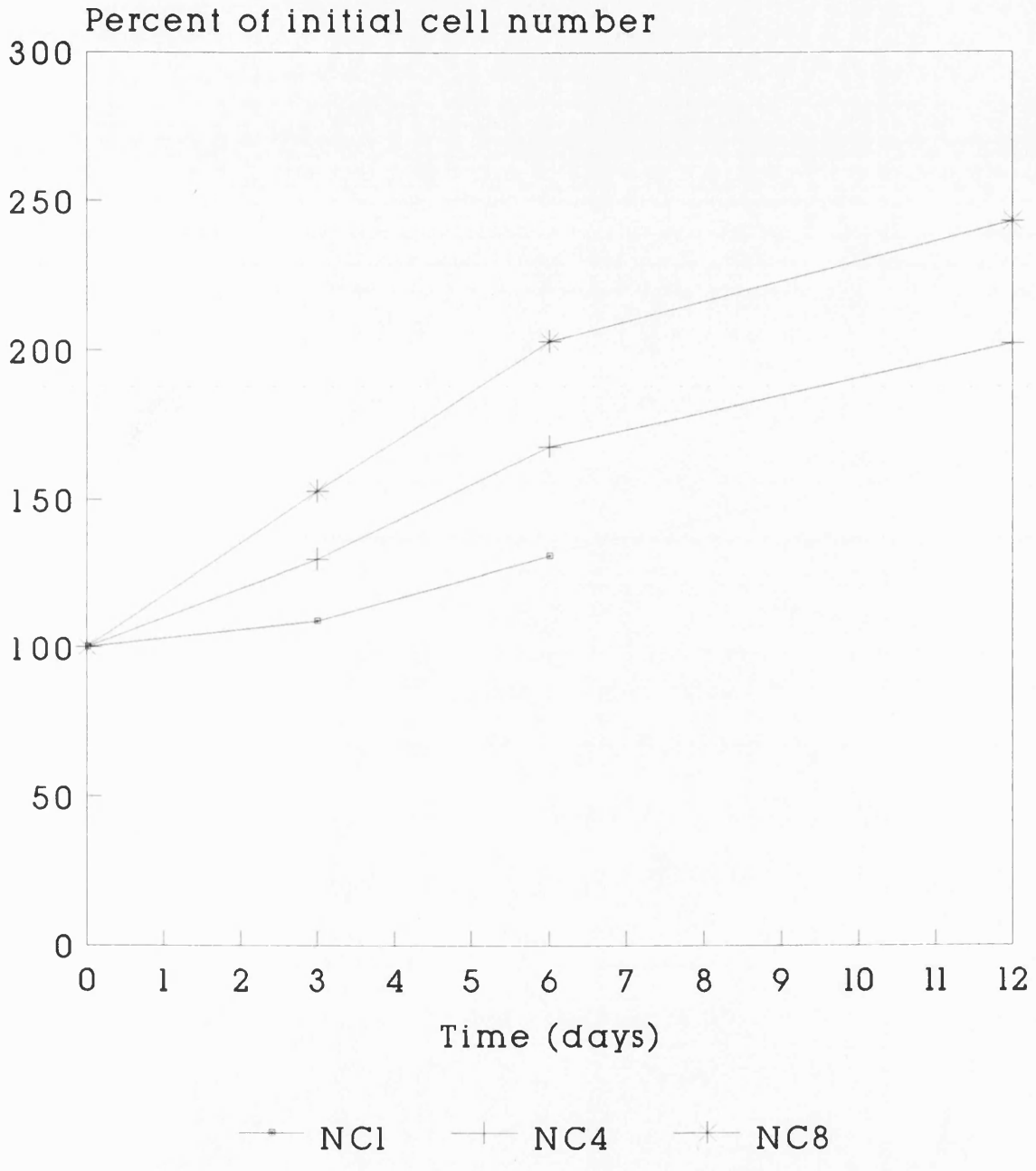


Figure 21. A suspension of control cells was plated out into 20 $4cm^2$ wells and grown in medium containing 1 mmol/L galactose. After 24hrs to allow cells to adhere five wells were trypsinised and counted (time 0). Further sets of five wells were counted at 2, 5 and 12 days. The final observations on line NC1 were lost because of infection of the culture.

Figure 22. Growth of Enzyme Deficient Fibroblasts in Galactose Enriched Media

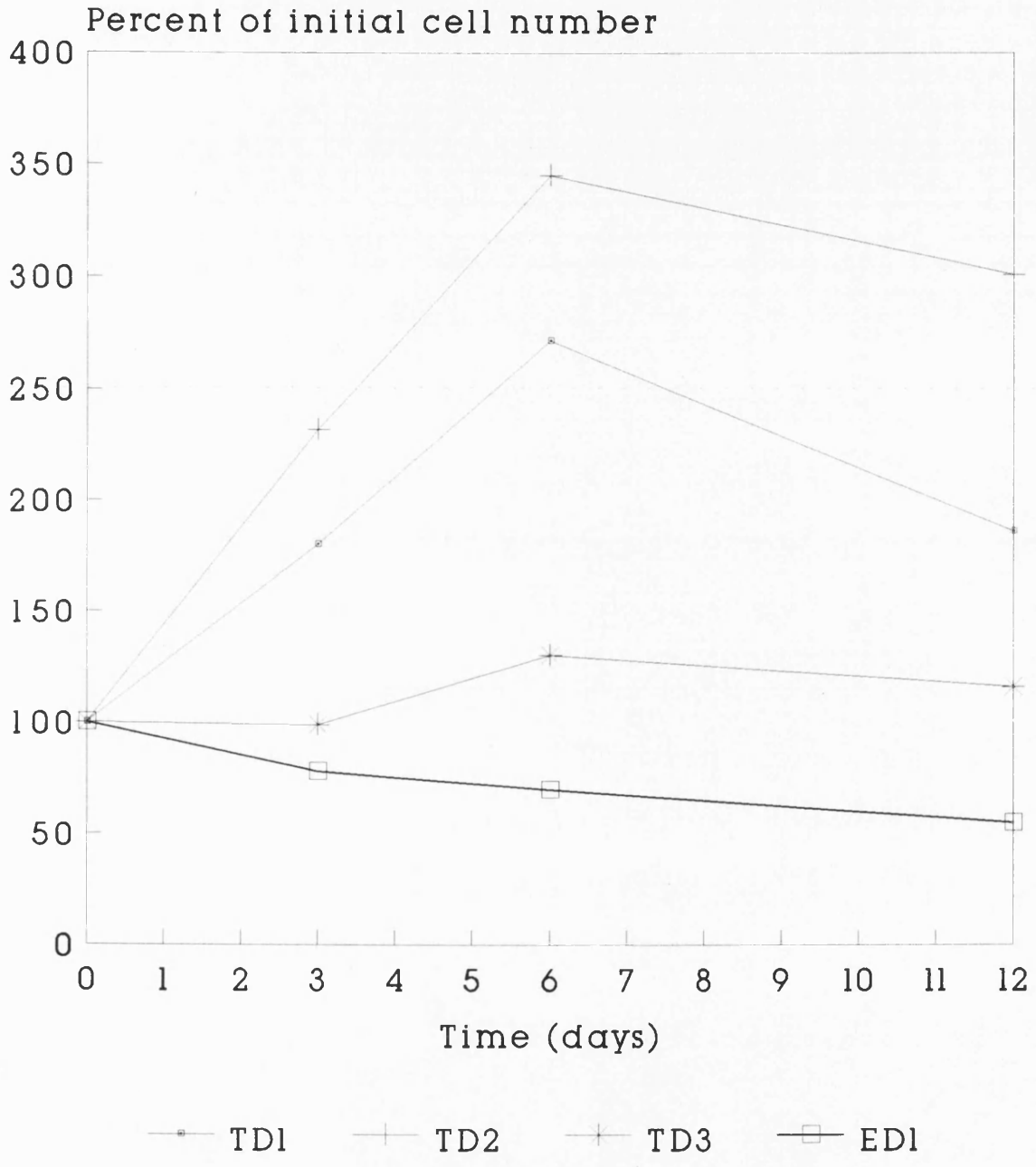


Figure 22. The conditions described in figure 21 were used to for transferase and epimerase deficient lines. TD = transferase deficient cell lines, ED = epimerase deficient.

Figure 23. CHO Cell Growth in Galactose Enriched Medium

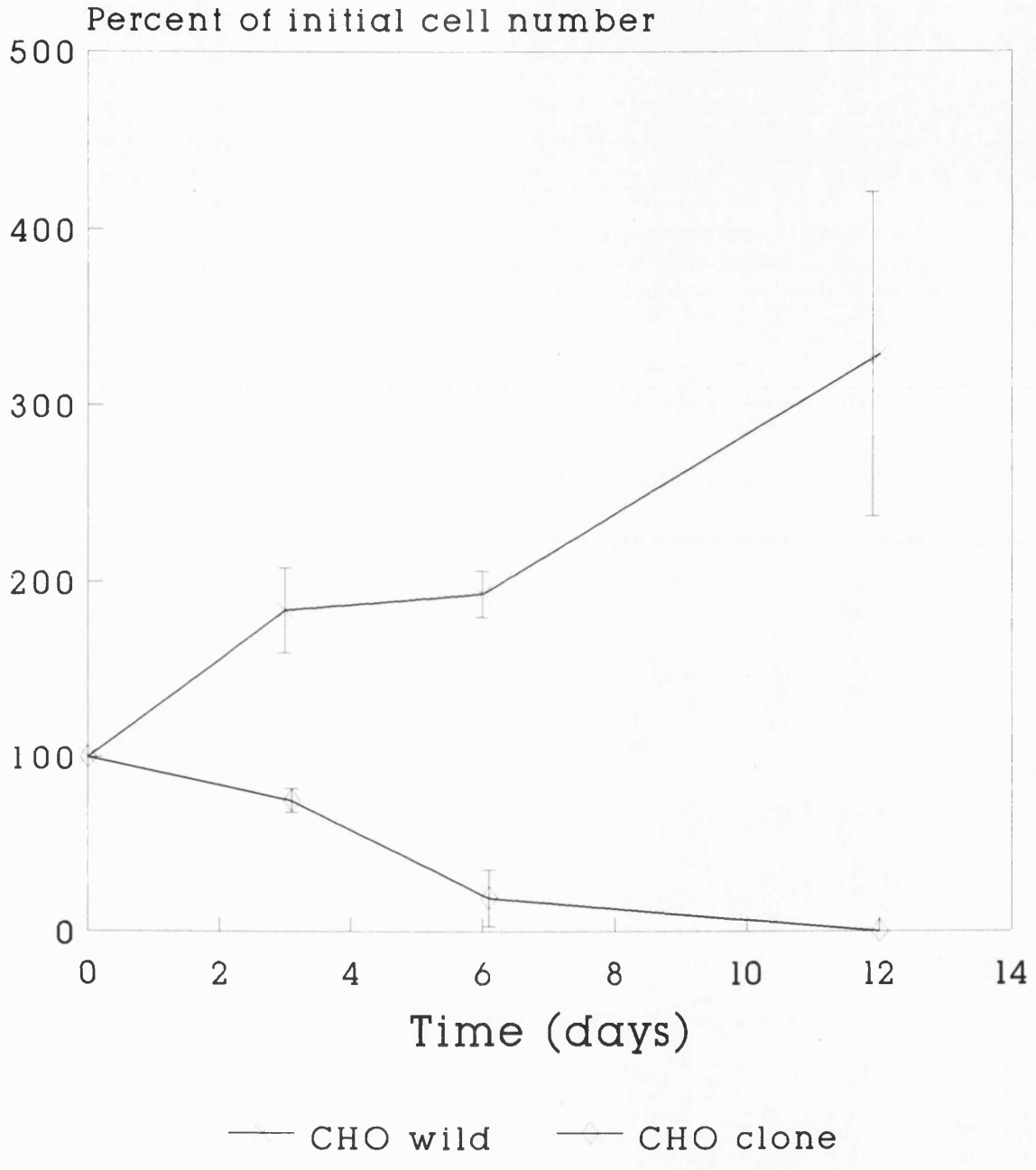


Figure 23. Both CHO cell lines were grown in galactose enriched medium as described in figure 21. Mean and standard error shown.

4.4.2 Leucine incorporation in Galactose Medium.

These experiments were similar to the leucine incorporation assessments carried out in normal medium, except that medium containing 0, 0.5, 1, 2.5 or 5 mmol/L of galactose was used. The mean and SD of each set of four observations and the total amount of cellular protein measured in each well are plotted in figures 24 - 30. These show two responses to increasing concentrations of galactose. Some lines (TD1, TD2, NC4, and NC5) show no change in leucine incorporation rate, while others (the epimerase deficient fibroblasts and lines TD3 and NC1) show decreases at higher galactose concentrations.

4.4.3 Photography of Effect of Galactose on Epimerase Deficient Cells.

Cells were grown in medium with 1 mmol/L galactose for 10 days. Photographs taken at 10 days are shown in figures 31-37. These show some areas of normal growth with healthy looking cells adhering to the flask surface plus an appropriately small number of cells rounded up as would be found in any growing population of cells. Other areas show a large proportion of cells rounded, cells with a granular appearance, very ragged clumps and areas where cells have died and floated off leaving a clear surface.

Figure 24. Leucine Incorporation Rate in Media with Galactose: Control Fibroblasts

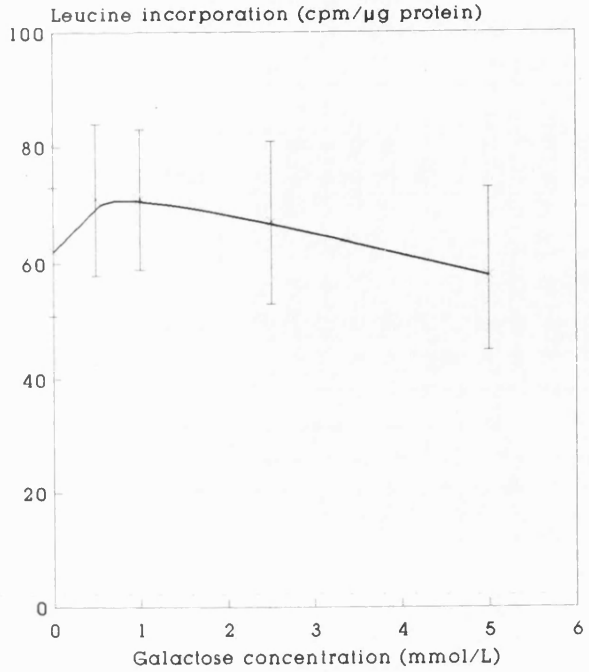
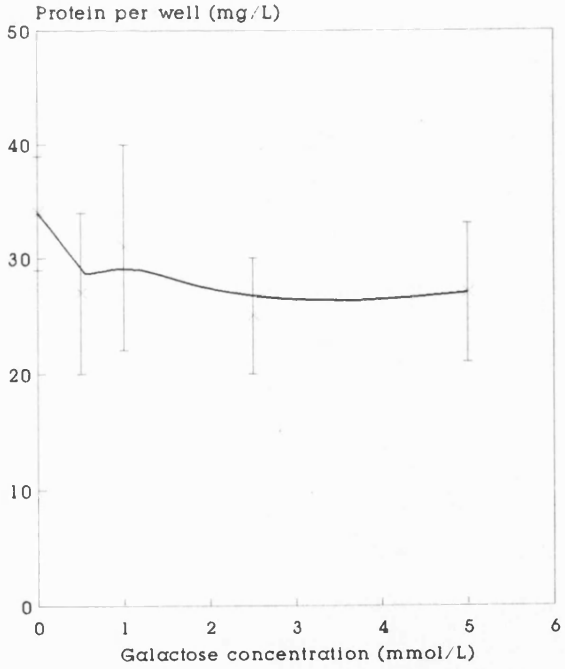
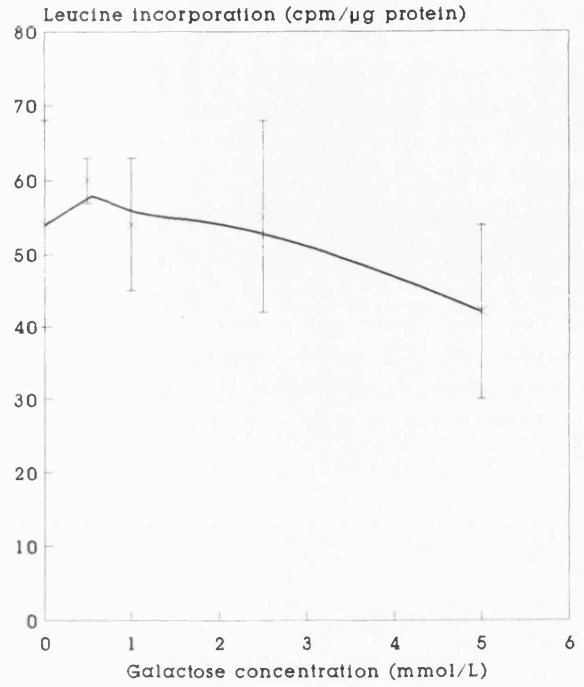


Figure 25. Protein per Well in Media with Galactose: Control Fibroblasts



A suspension of control fibroblast lines was plated out into 25 4cm^2 wells of a multiwell plate. In five wells galactose free medium was used, and in four other groups of five wells medium supplemented with 0.5, 1, 2.5 or 5 mmol/L galactose was added. In addition the medium added to 4 of each set of 5 wells contained 1 µCi/ml of ^{3}H leucine, the remaining well being designated a blank. After 6hrs the amount of radioactivity incorporated and the level of protein remaining in each well were assessed and the incorporation rate per unit protein calculated.

Figure 26. Leucine Incorporation Rate in Media with Galactose: Transferase Deficient fibroblast line TD1



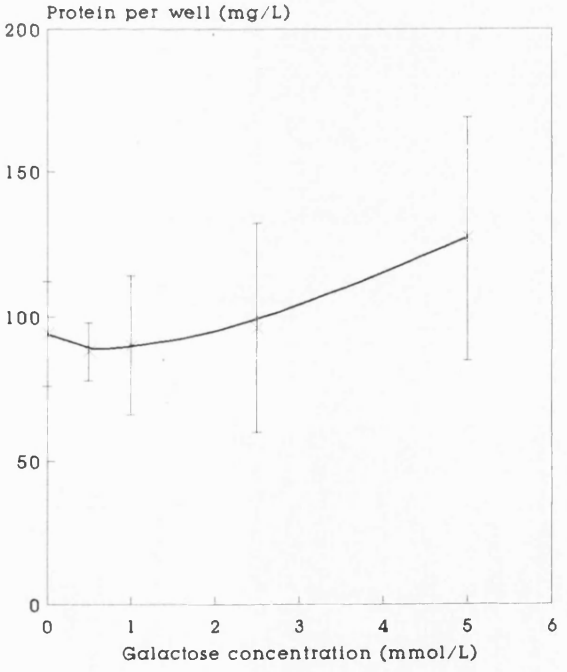


Figure 26. The leucine incorporation rate as a function of galactose concentration was assessed for line TD1 as described in figures 24 and 25.

Figure 27. Leucine Incorporation and Protein per Well in Media with Galactose: Transferase Deficient Line TD2

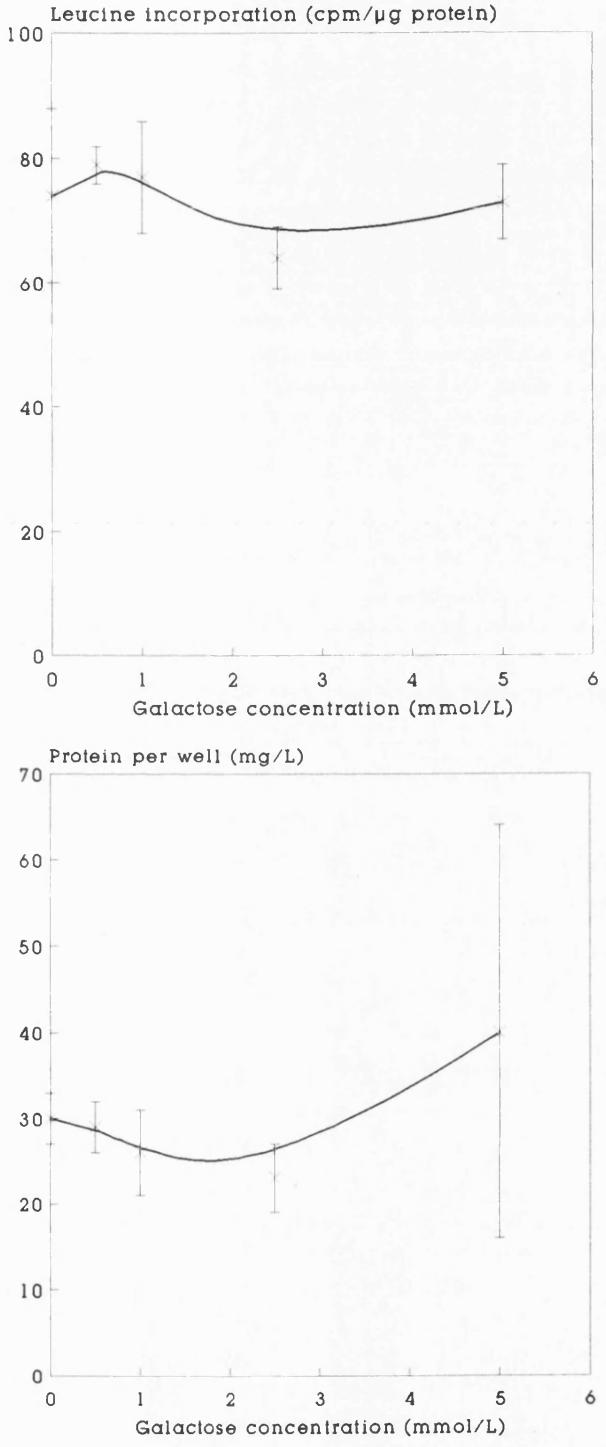


Figure 27. The leucine incorporation rate as a function of galactose concentration was assessed for line TD2 as described in figures 24 and 25.

Figure 28. Leucine Incorporation and Protein per Well in Media with Galactose: Transferase Deficient Line TD3

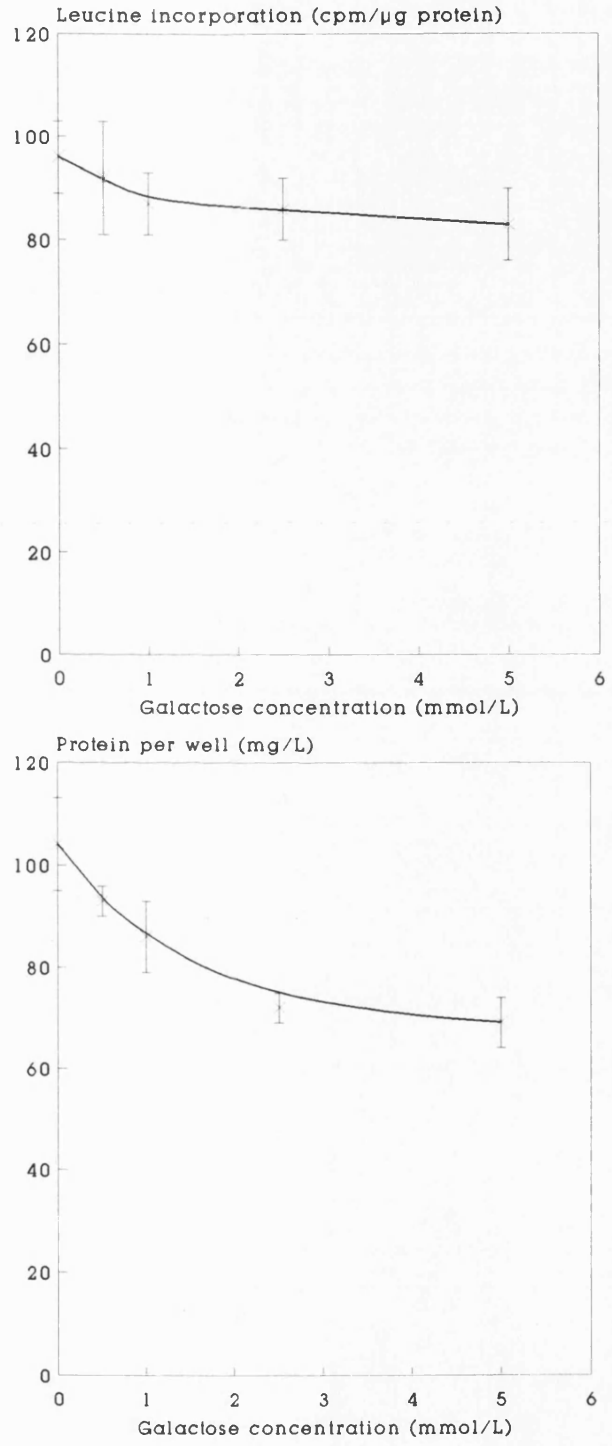


Figure 28. The leucine incorporation rate as a function of galactose concentration was assessed for line TD3 as described in figures 24 and 25.

Figure 29. Leucine Incorporation in Media with Galactose: Epimerase Deficient Line ED1

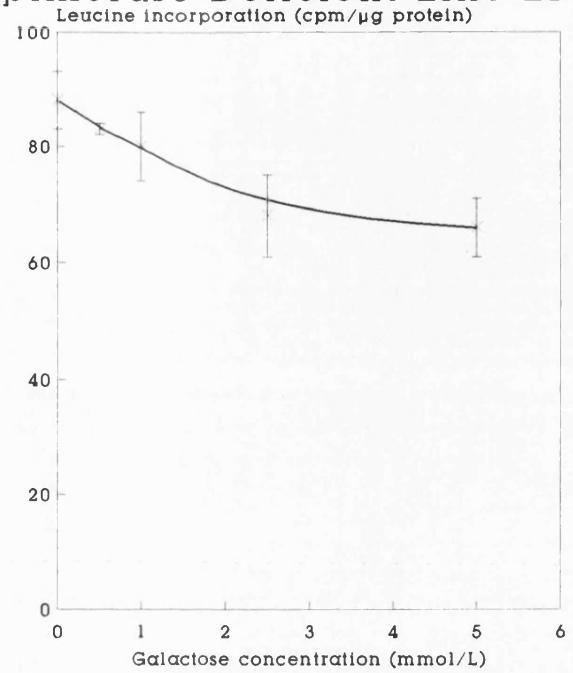


Figure 30. Protein per Well in Media with Galactose: Epimerase Deficient Line ED1

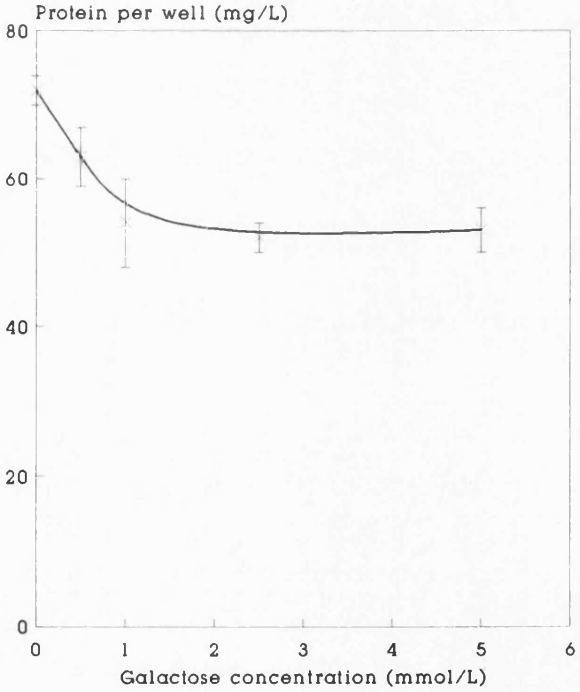


Figure 29 & 30. The leucine incorporation rate as a function of galactose concentration was assessed for line ED1 as described in figures 24 and 25.

Figure 31. Epimerase deficient fibroblasts were grown in medium supplemented with 1 mmol/L of galactose. After 10 days a wide range of microscopic appearances was observed. In this section cells appeared identical to control cells, with good adhesion and a small proportion of cells rounded up as occurs in normal division.

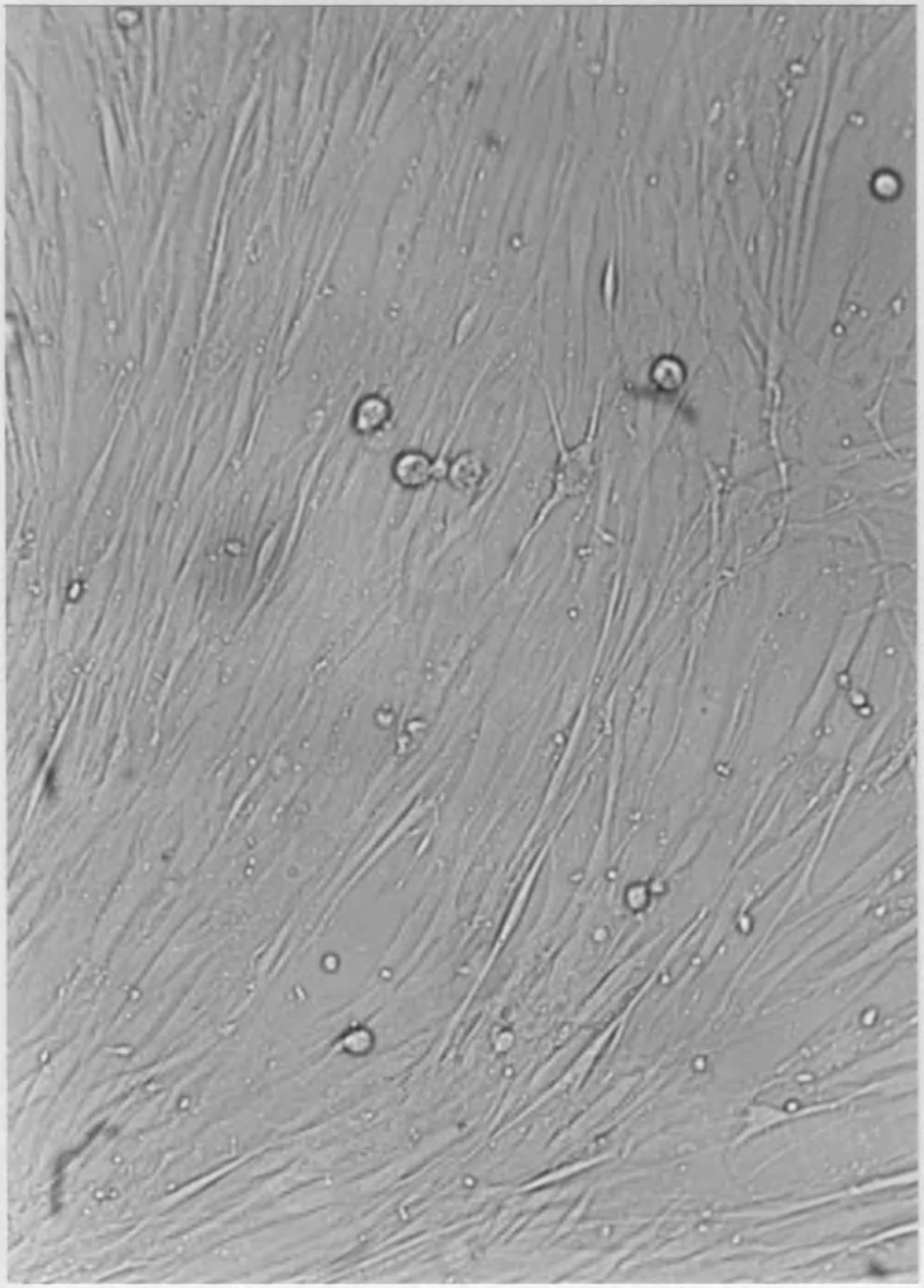


Figure 32. Another section of the monolayer also has a normal appearance.

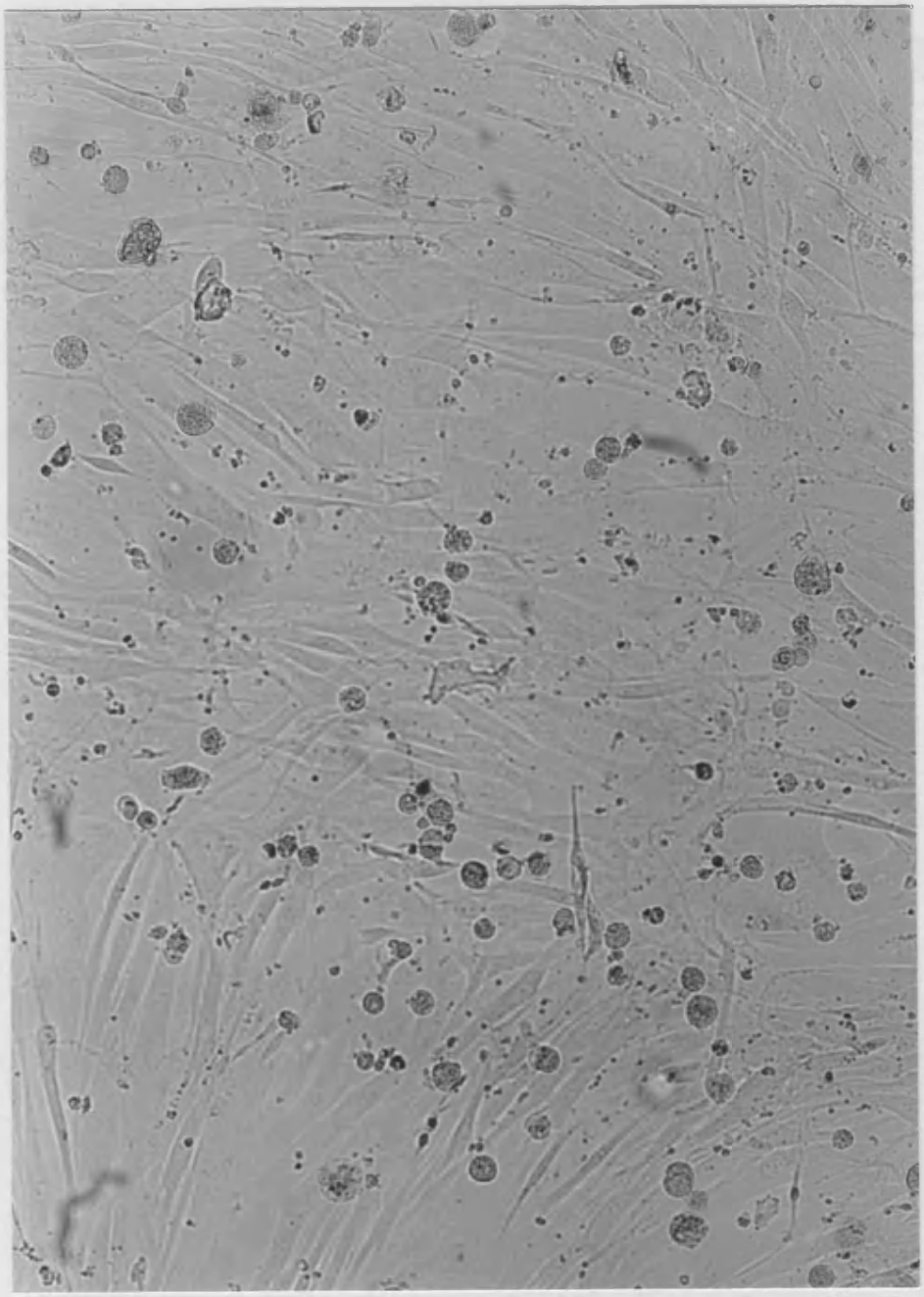


Figure 33. This section shows an abnormally high number of cells rounded up, demonstrating a toxic effect resulting in failure of adhesion. The rounded cells also have an abnormal granular appearance.

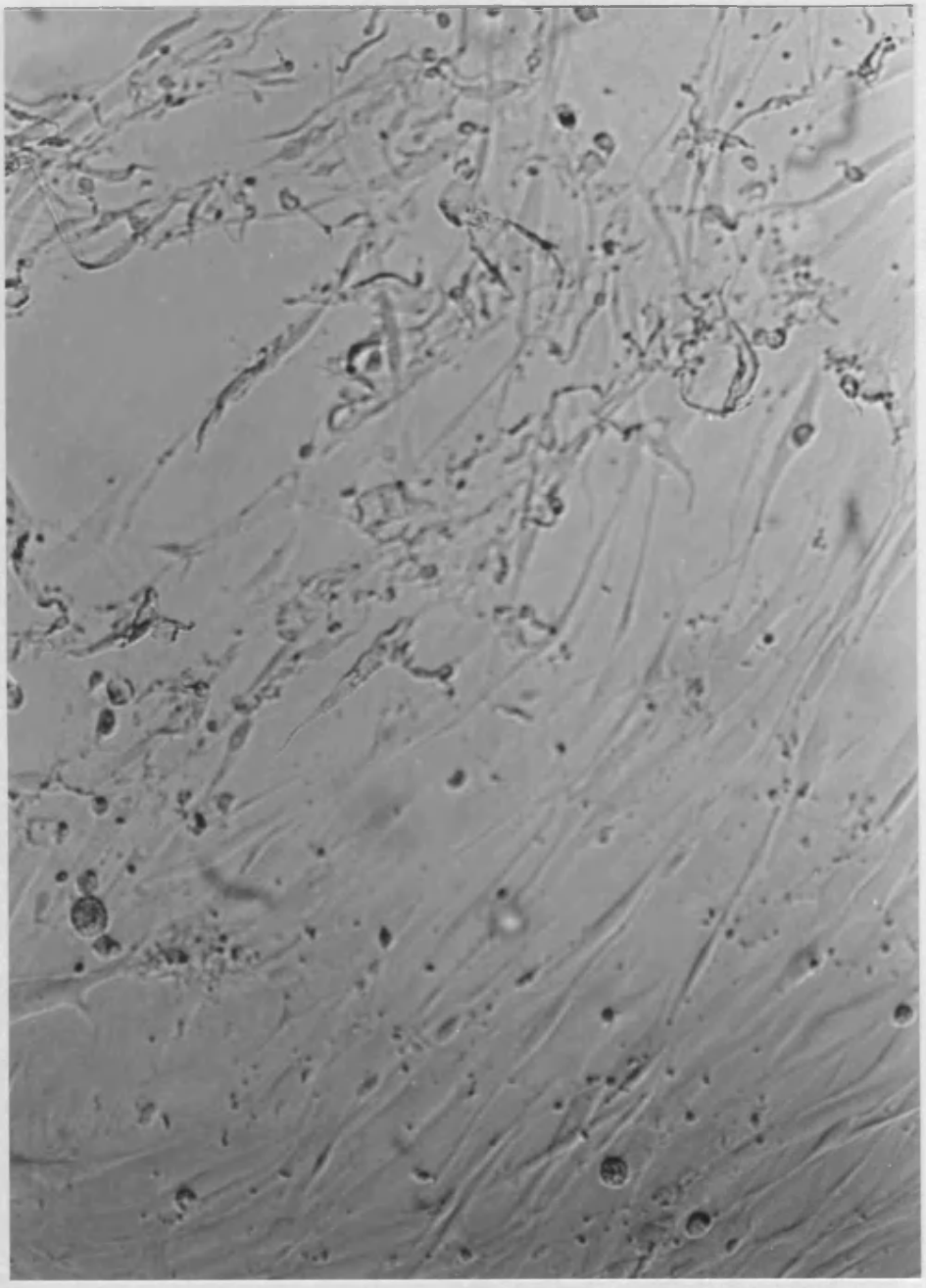


Figure 34. This shows an area of fairly healthy looking confluent cells graduating into a patch of ragged, unhealthy looking cells. They appear irregular and shrunken, and are not adhering, but have not rounded up.

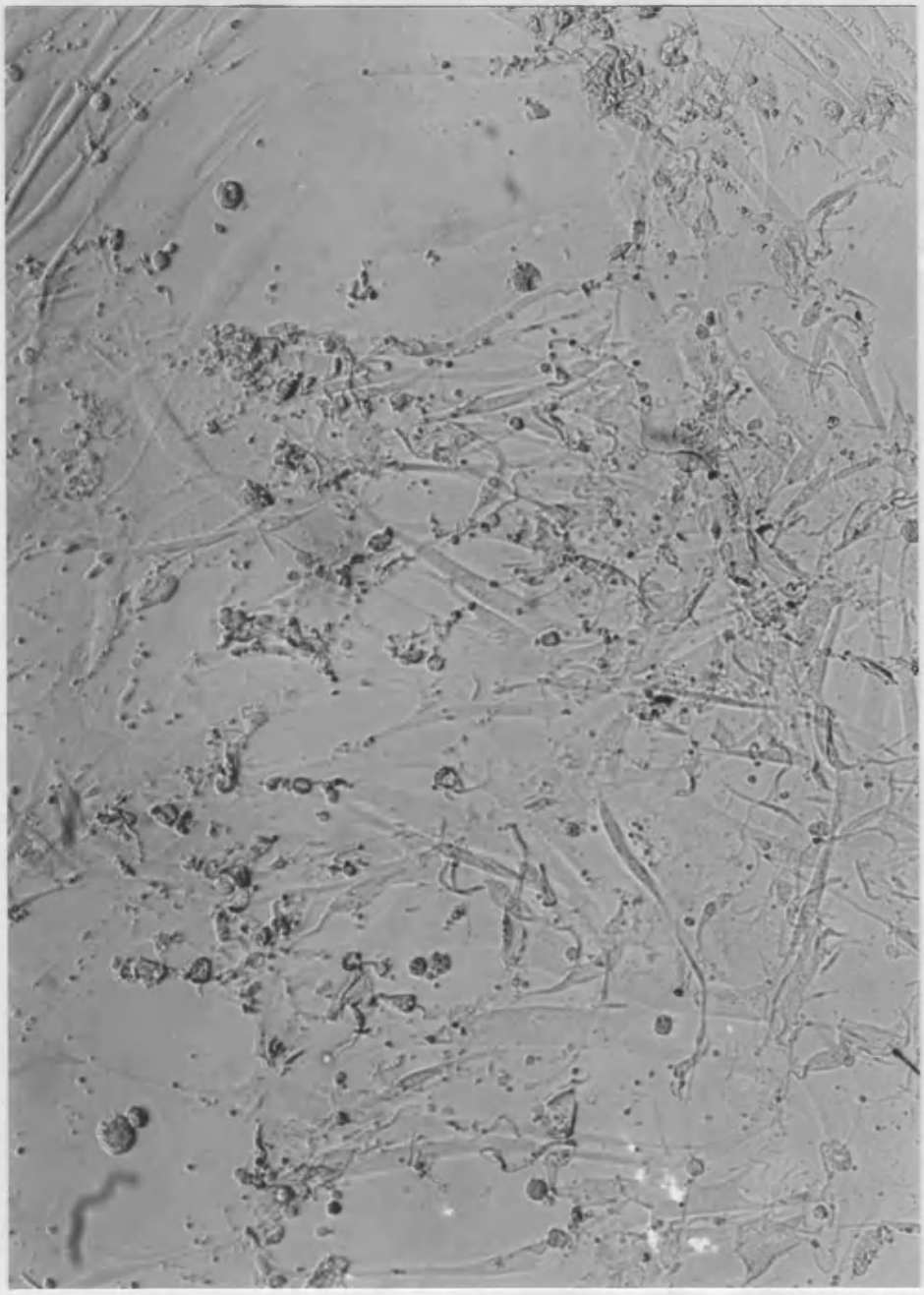


Figure 35. This shows an area where most of a population of healthy cells have been lost and many of the remaining are irregular and mis-shapen. A large number of particles of cellular debris are evident.

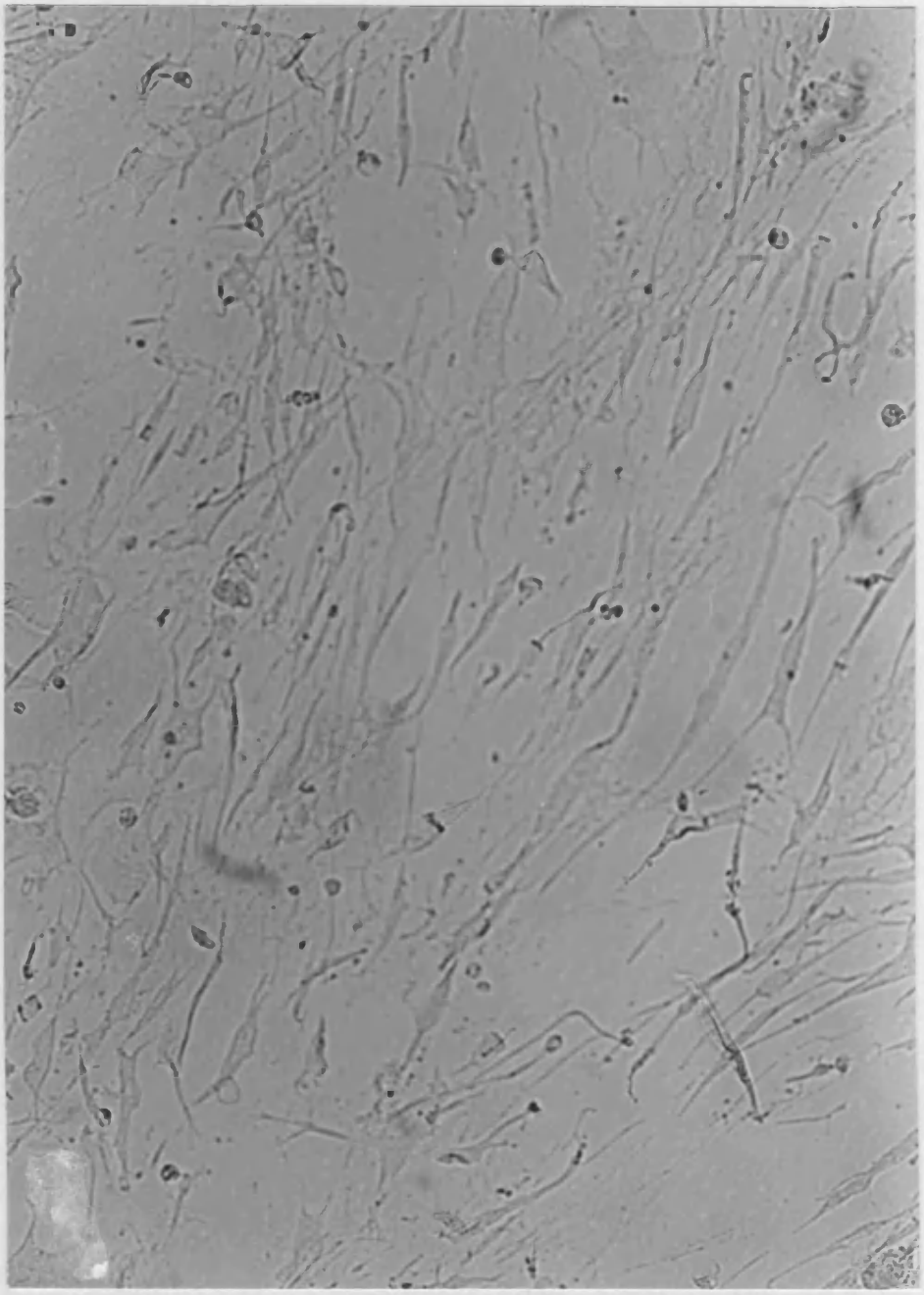


Figure 36. Some patches of cells became abnormally thin and spindly without acquiring a granular or ragged appearance.

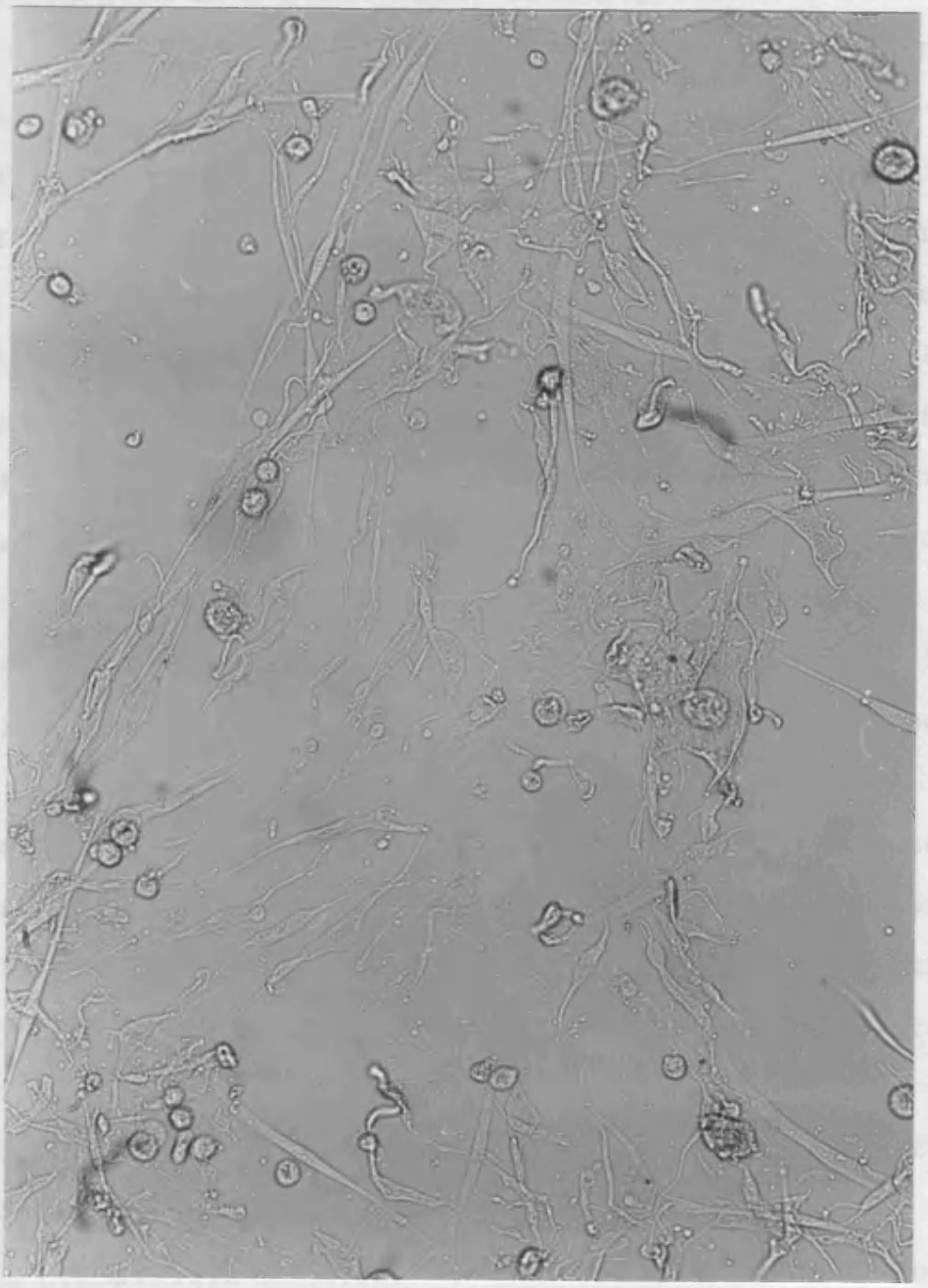


Figure 37. In places most cells had died leaving behind a few grossly abnormal remnants.

4.4.4 Growth in Galactose Media - Discussion

This section investigates the growth of cells in media supplemented with galactose. Some reports have been published of the effects of such media on transferase deficient cells, but none on epimerase deficient lines. Galactose is known be toxic to transferase deficient lines, but there are differences in the literature over the severity and rapidity of this effect. All authors used either 5 or 5.5 mmol/L galactose. Krooth (1960) found that cells grew until the 6th day then the population levelled off or decreased slightly, and Tedesco (1979) observed that cells exhibited noticeable changes at 1 week, deterioration over 1 to 2 weeks and irreversible damage after 2 to 3 weeks, but does not quantitate these observations. In contrast Pourci (1990) found that under very similar conditions cells stopped growing in 2 days and were all dead by 7.

The observations on cell numbers in galactose enriched media (figures 21, 22 and 23) show three types of curve, with every cell line of a given type rigorously following the same curve. All control cell lines show a steady increase with time. This confirms the observations of the above authors that galactose is capable of acting as an energy source for control cells.

All transferase deficient lines have increased in number by day 6 but all decrease between days 6 and 12. These results are therefore in agreement with those of Tedesco and Krooth described above, and in contradiction with those of Pourci. It is difficult to reconcile this differance unless it is a result of a disparity in culture conditions or materials not

described by Pourci. Contamination of the galactose used by Pourci is unlikely, since control fibroblasts grew in the medium.

The third type of curve evident is a steady decline, shown by both epimerase deficient lines, that for CHO cells being particularly marked in comparison to controls (fig. 23). Thus there is a difference between the cytotoxic effects on epimerase and transferase deficient lines. It was formerly considered that gal-1-P was the cytotoxic agent (eg Gitzelmann 1980) but Pourci found that addition of inosine to the culture medium protects transferase deficient cells from the effects of galactose, despite high levels of gal-1-P. In erythrocytes, the levels of UDP gal are raised in epimerase deficiency (Henderson 1983) and lowered in transferase deficiency (Ng 1967, Xu 2989b). The very different results for TD and ED cell lines in figure 22 suggest that different cytoxic processes are affecting the two cell types, possibly the alteration of UDP gal levels in opposite directions mentioned above.

The next series of experiments measured leucine incorporation as a function of galactose concentration. Cells were plated out at approximately similar multiplicates for any one line, so the total amount of protein per well is also represented in the results, otherwise if most cells died but the remainder were incorporating protein at a normal rate no abnormality would be apparent in the leucine incorporation results. In some results, the protein per well is highly variable (for instance, note the large error bars in figures 26 and 27). However, the leucine incorporation rates which were calculated

from these results have smaller error bars, suggesting that this is not an analytical error, but that replicates in plating out were variable, and that once results were normalised for protein, errors were acceptable. Possible causes include sedimentation of the cell suspension used for plating out, or formation of clumps of cells.

Figures 24 and 25 show that control fibroblasts were unaffected by concentrations of galactose up to 5 mmol/L, as would be expected from the results in section 4.4.1. Of the transferase deficient lines two showed no change and one a significant decrease in leucine incorporation and amount of protein per well. It may seem surprising that lines TD1 and TD2 were unaffected, but they were exposed to galactose for 6 hrs, and in section 4.4.1 it was shown that full toxic effects are not exhibited until after seven days of exposure. Cell line TD3 decreased its rate of leucine incorporation by 12% when the galactose concentration was increased from zero to 5mmol/l, showing that this cell line is more sensitive to the toxic effects of galactose over this time period.

The line ED1 also showed a significant drop in both parameters measured. Kalkar (1965) hypothesised that a completely epimerase deficient organism would be a galactose auxotroph, ie, it would require supplementation with exogenous galactose for normal growth. The cell line ED1 did have reduced growth in normal medium. If Kalkar's hypothesis applied to the line ED1 it is possible that supplementation with small amount of galactose in vitro (and by extension in vivo) would result in improved growth, whereas higher amounts would induce toxic effects from an accumulation of metabolites. Only the latter

effect is evident. Thus either galactose is only toxic to these cells, or the level of supplementation required for benefit is so low as to be unobservable in these experiments. Various authors have published photographs of the effect of galactose on transferase deficient cells (eg Miller 1968, Pourci 1990) but none have illustrated the effect on epimerase deficient cells. Figures 31 to 37 show a number of abnormal features. These include a high proportion of cells rounded up, too high to represent normal levels of division, and unnatural granular appearance of the cells, a large amount of cellular debris from dead cells, vaculolation and loss of adhesion. These effects are similar to those described in transferase deficient cells but this is not evidence for a common mechanism since such effects are a final common pathway to a large number of cytotoxic processes.

4.5 Galactose in Cells.

These experiments assessed the galactose content of cultured cells in order to establish whether cells with abnormalities of galactose metabolism also have different amounts of galactose incorporated in the cells. Three techniques were used: gas chromatography of monosaccharides in whole cell preparations, Western blots of cell membrane preparations with a galactose specific detection system, and a series of experiments with ricin lectin attached to fluorescein isothiocyanate; this was bound to cell membranes and the resulting fluorecence was photographed and quantitated.

4.5.1 Gas Chromatography.

Hydrolysis of cell glycoproteins and gas chromatography of the resultant monosaccharides gave good reliable separation and allowed identification of the following sugars: mannose, galactose, glucose, ribose, N-acetyl glucosamine, N-acetyl galactosamine and sialic acid. Peak areas were assessed by the integration function of the gas chromatograph data handler. A sample trace is shown in figure 38.
The results of these analyses, expressed as nanomoles carbohydrate per milligram protein, are shown in Table 4. The duplicate figures for each cell line represent duplicates of the entire procedure of cell culture, cell preparation and lyophilisation, protein analysis and gas

Cell line	Man	Gal	Glc	GlcNac	GalNAC	SA	Ribose
NC6	0.71	0.76	13.9	0.53	--	0.29	0.82
	0.65	0.70	13.5	--	--	0.40	0.70
NC8	1.3	0.66	14	--	--	0.16	1.2
	0.92	0.83	12.8	1.2	0.33	0.42	0.92
NC9	1.1	1.2	10.5	0.85	0.71	0.57	1.4
	0.87	1.2	8.0	0.63	0.63	0.44	1.1
NC10	0.66	0.66	11.0	0.50	0.50	0.34	0.47
	0.61	0.65	11.0	0.58	0.19	0.32	0.39
ED1	0.67	0.55	3.1	0.50	0.39	0.12	0.83
	0.61	0.55	14.3	0.39	--	0.28	0.50
TD1	0.50	0.38	3.0	0.23	--	0.15	0.35
	0.59	0.41	3.4	0.27	--	0.14	0.45
TD2	1.6	1.22	15.00	--	--	0.78	0.89
	0.82	0.72	8.1	0.36	0.27	0.18	0.36
TD3	0.66	0.46	4.7	0.32	--	0.24	0.51
	0.58	0.39	4.3	0.29	--	0.18	0.47
CHO	0.50	0.25	0.25	--	--	--	0.44
wild	0.60	0.20	0.13	--	--	0.06	0.46
CHO	0.59	0.36	1.20	--	--	0.27	0.64
clone	0.58	0.33	0.37	--	--	0.12	0.83

Table 4 Nanomoles of carbohydrate per milligram protein in hydrolysed cell preparations. Duplicate measurements made. Man = mannose Gal = galactose Glc - glucose GlcNAC = N-acetyl glucosamine GalNAC = N-acetylgalactosamine SA = Sialic Acid

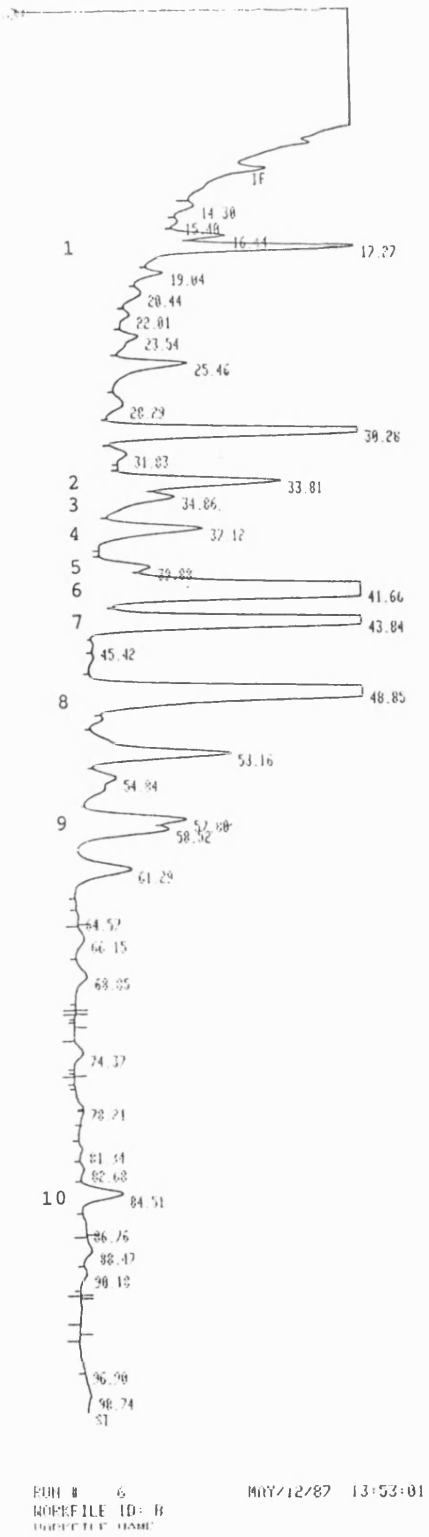


Figure 38. Gas chromatography of cell lysates. Lyophilised cell preparations were hydrolysed with methanolic HCl then silylated. The product was analysed by gas chromatography on an SE-30 column ramped between 140 and 200°C at 0.5°C/minute. Peaks resolved included 1 ribose, 2 mannose, 3,4,5, galactose, 6,7 glucose, 8 mannitol, 9 N-acetyl glucosamine and 10 sialic acid.

chromatographic analysis, not merely double analysis of one derivitised sample. The results for all normal control lines were combined and the means and standard deviation calculated. This was repeated for transferase deficient lines. The resultant values were subjected to Student's T-Test. These are shown in Table 5.

	Galactose(μg) protein(μg)	Sialic acid+galactose Mannose (μg)	Galactose Mannose(μg)
Control (n=8)	0.83 ± 0.21	1.46 ± 0.37	1.00 ± 0.22
Transferase deficient (n=6)	0.60 ± 0.32	1.00 ± 0.13*	0.77 ±0.08*

Table 5. Carbohydrate composition of cells. * Means of transferase deficient and control lines significantly different ($P < 0.05$)

4.5.2 Western Blots of Cell Membrane Preparations.

After electrophoresis in a polyacrylamide gel proteins were transfered to nitrate paper. The gel was then stained with Coumassie Brilliant Blue dye to allow assessment of the protein electrophoresis. A photograph of a gel is shown in figures 39. This shows good protein separation (i.e. correct gel preparation), progress of the protein front almost to the bottom of the gel (i.e. appropriate running time), good linear protein travel and proteins distributed fully across each

track (i.e. no air bubbles trapped in sample wells or at the bottom of the gel).

The nitrate papers were also photographed at the end of the blotting procedure. The molecular weight marker strip was stained with Coumassie Brilliant Blue and the remaining strips with the galactose specific lectin based stain for test strips and this stain plus galactose for controls.

A photograph of a blotted nitrate paper is shown in figure 40. Normal control fibroblasts show a large number of discrete bands on top of an increased background stain when compared to controls. Epimerase deficient fibroblasts show an identical pattern of bands and background stain, but at a lower density. This shows that epimerase deficient fibroblasts grown under the conditions described have galactose-containing glycoproteins in their cell membranes.

For the wild type Chinese Hamster Ovary cells stained bands are also evident over the same molecular weight range as for fibroblasts but with a different pattern. Epimerase deficient CHO cells show a very different pattern. The first (i.e. highest molecular weight) band of the wild type cells is clearly present to a similar degree but the subsequent banded pattern is completely absent. There is a general increase in background staining, although to a lower extent than with the wild type cells.

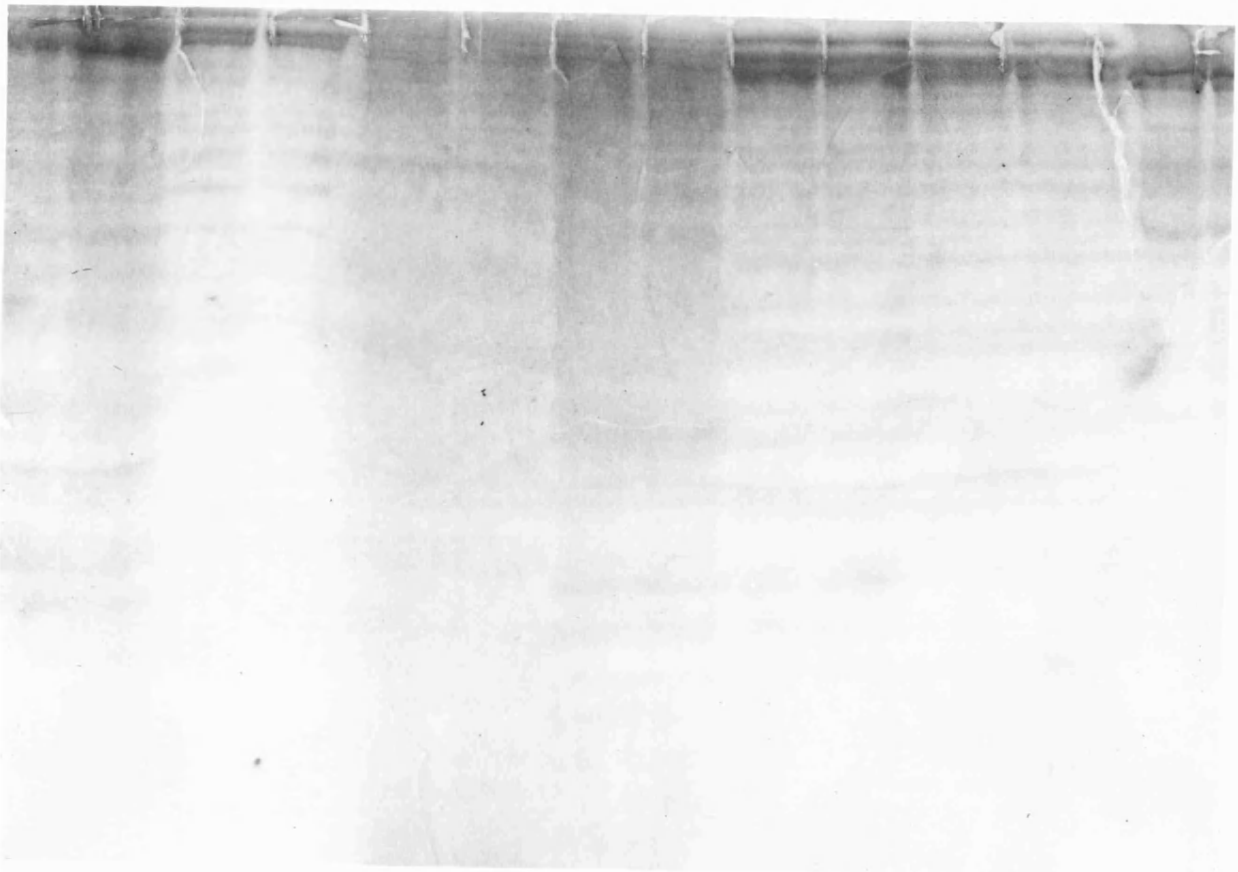


Figure 39. Western Blot stage 1: electrophoresis.
Solubilised cell preparations were subjected to electrophoresis in an 8% SDS-polyacrylamide gel. After electrotransfer to cellulose nitrate paper the gel was stained with Coumassie Brilliant blue, dried and photographed.

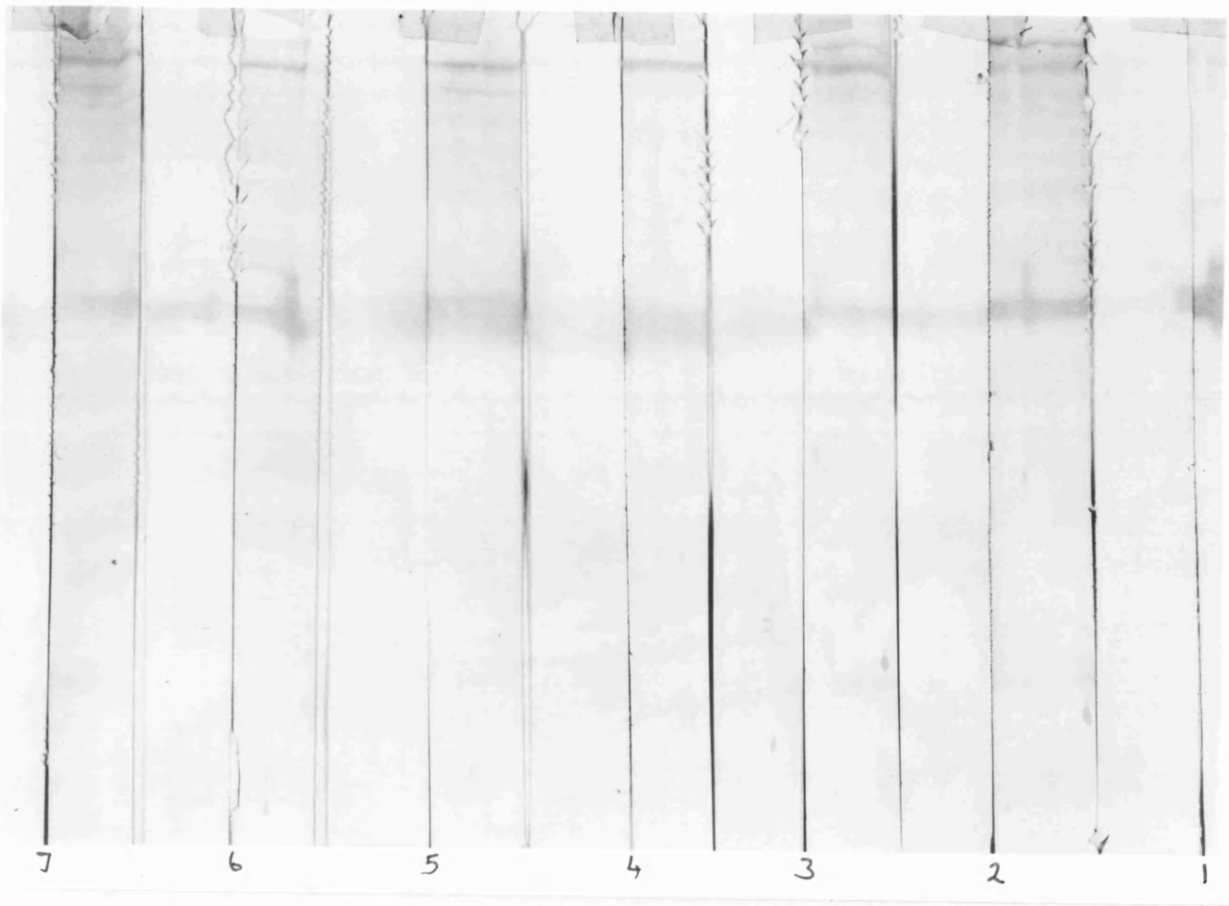


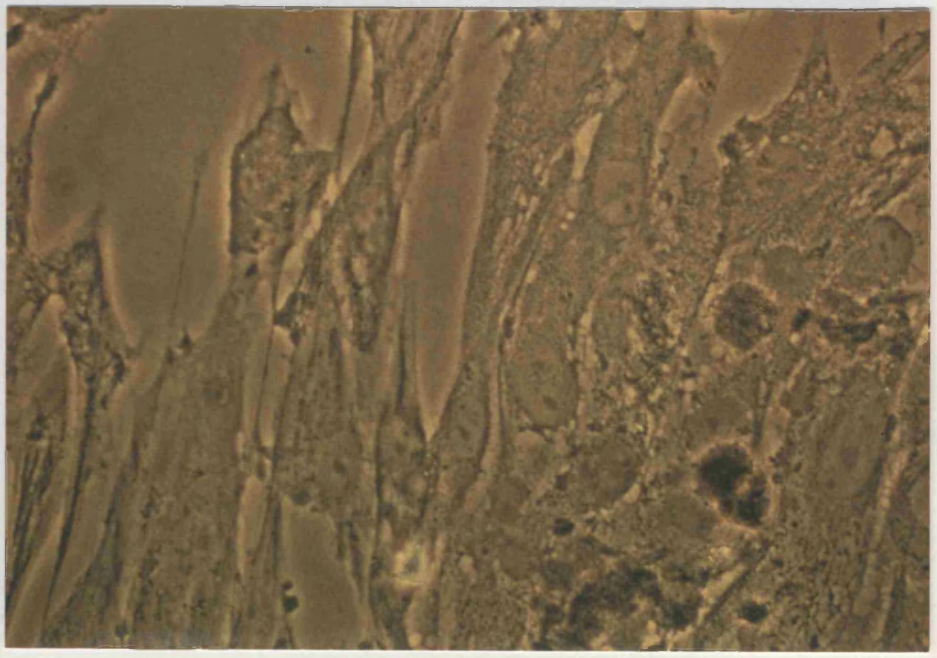
Figure 40. Western Blot stage 2: solublised cell preparations were electrophoresed on SDS-polyacrylamide gel, blotted onto cellulose acetate paper and detected with a galactose specific lectin preparation. 1: normal red cell ghosts, 2: normal control fibroblasts, 3: normal control fibroblasts, 4: epimerase deficient CHO cells, 5: wild CHO cells, 6: epimerase deficient fibroblasts, 7: normal control fibroblasts.

4.5.3 Examination and Photography of cells stained with FITC linked lectin.

Slides were initially examined under phase-contrast and visible light illumination. This showed adequate cell densities on all slides. Cell integrity was assessed by examining for trypan blue staining. On all slides the vast majority of cells were not heavily stained, showing that the staining procedure had not visibly damaged the cells. Examination of test slides under ultra-violet illumination showed diffuse green fluorescence restricted to cells. Control slides (those where galactose was added to the stain) showed no fluorescence. Results of the examination are shown in Table 6.

Cell Line		Result
NC 8	test	fluorescence
	control	no fluorescence
NC 11	test	fluorescence
	control	no fluorescence
NC 12	test	fluorescence
	control	no fluorescence
TD 1	test	fluorescence
	control	no fluorescence
TD 2	test	fluorescence
	control	no fluorescence
TD 3	test	fluorescence
	control	no fluorescence
ED 1	test	fluorescence
	control	no fluorescence
CHO wild	test	fluorescence
	control	no fluorescence
CHO clone (ordinary medium)	test	very low fluorescence
	control	no fluorescence
CHO clone (dialysed medium)	test	low fluorescence
	control	no fluorescence

Table 6. Staining of cells with FITC linked lectin



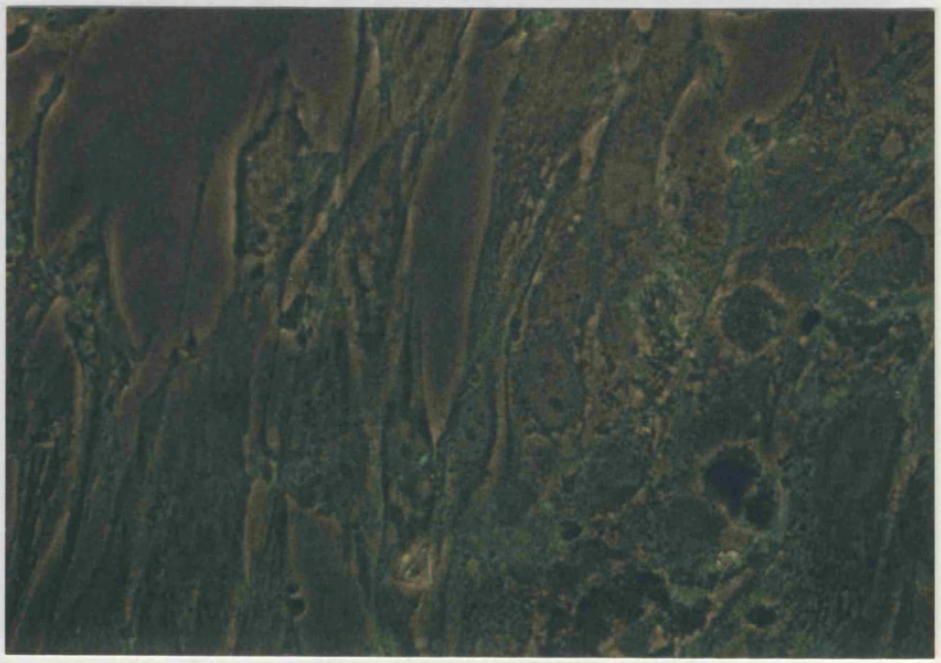


Figure 41 to 48. Cells were grown on sterile glass coverslips then stained either with fluoroscein linked lectin solution (tests) or fluoroscein linked lectin solution plus galactose (controls). The stained slides were examined microscopically and photographed under phase contrast illumination, ultra-violet light, or a combination of the two. On this page figs. 41 and 42: normal control cell line NC1 under combined and UV illumination.

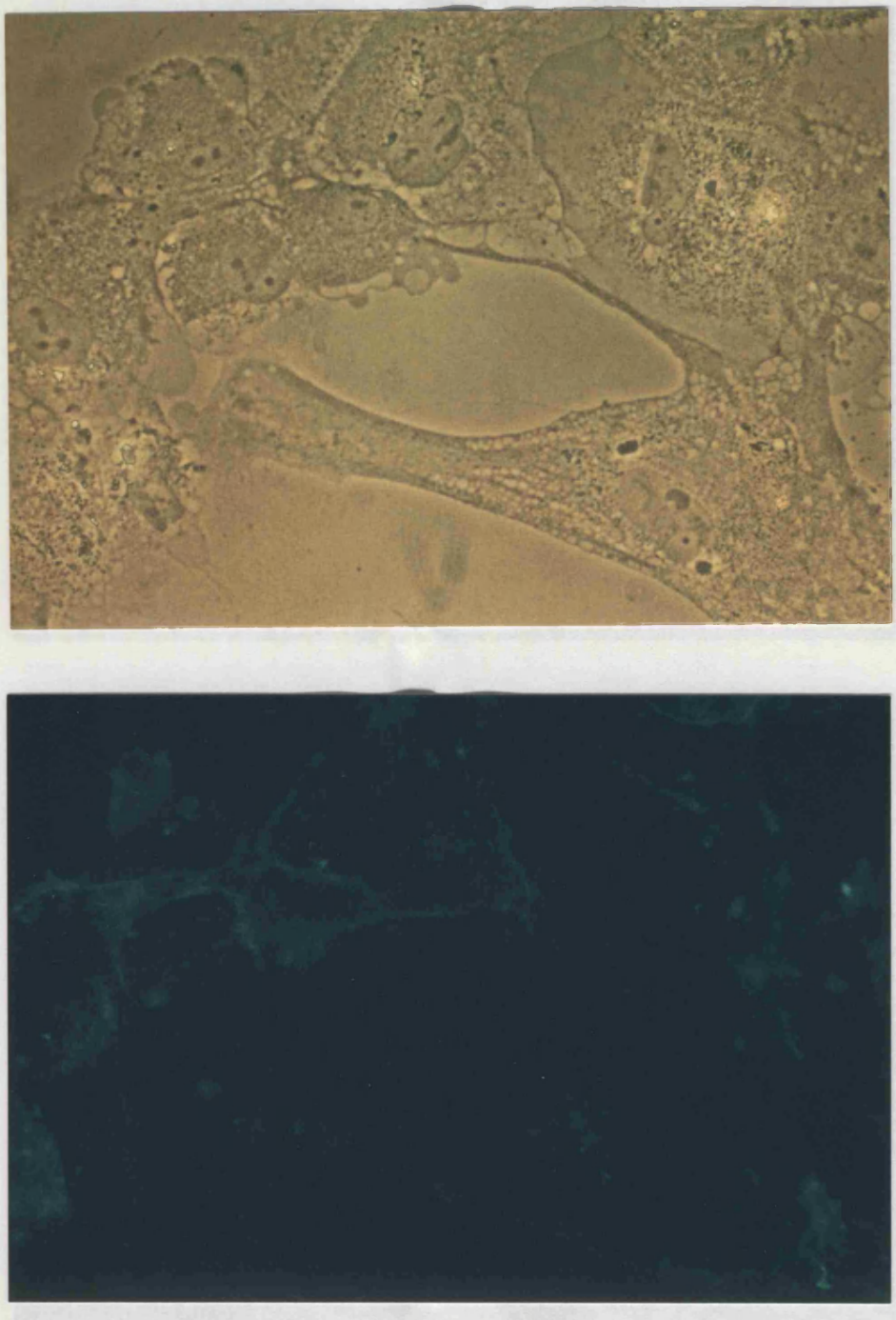


Figure 43. An area of control cell line NC4 under phase contrast and UV illumination.

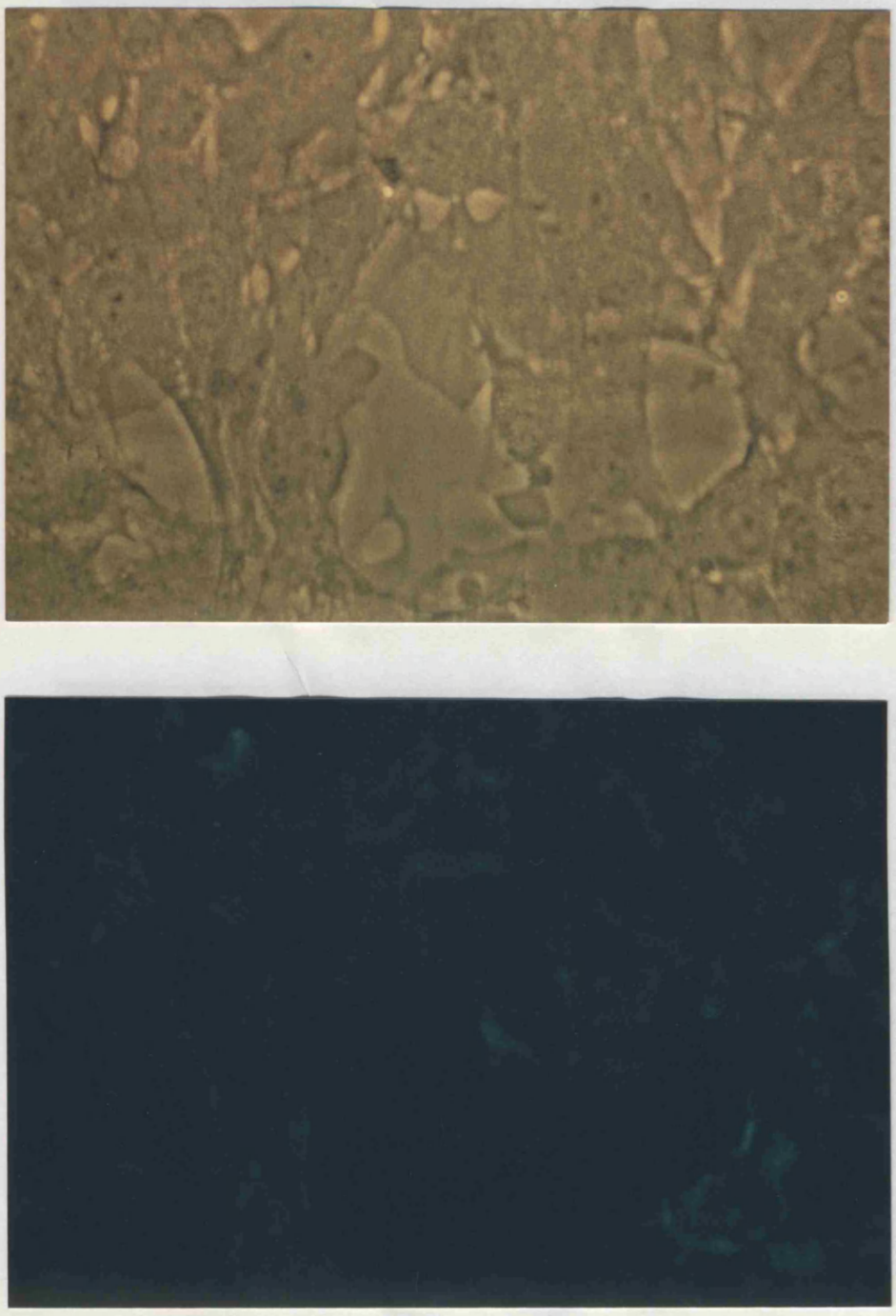


Figure 44. An area of transferase deficient cell line TD1 under phase contrast and UV illumination.

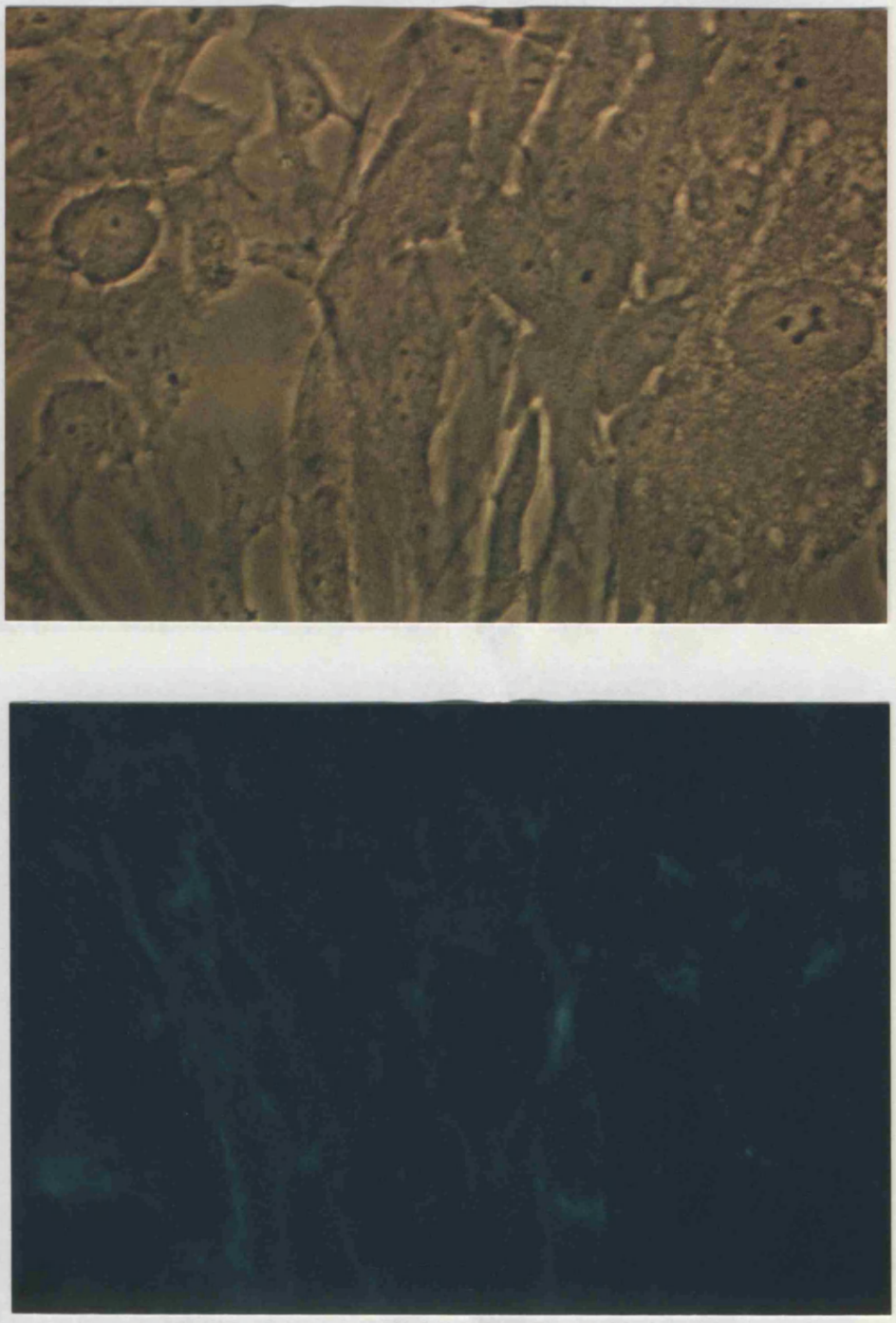
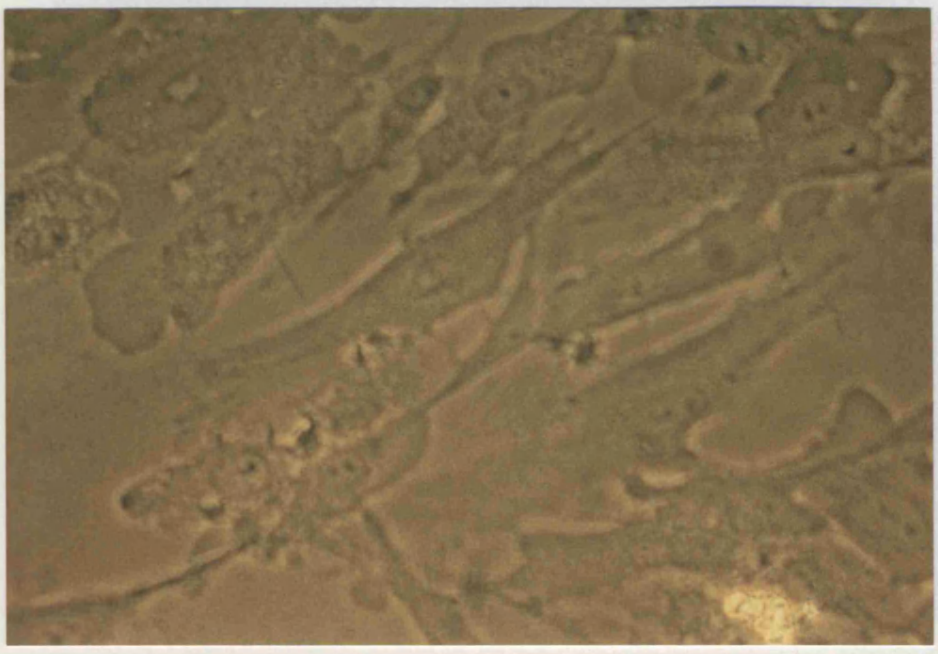


Figure 45. An area of epimerase deficient fibroblast line ED1 under phase contrast and UV illumination.



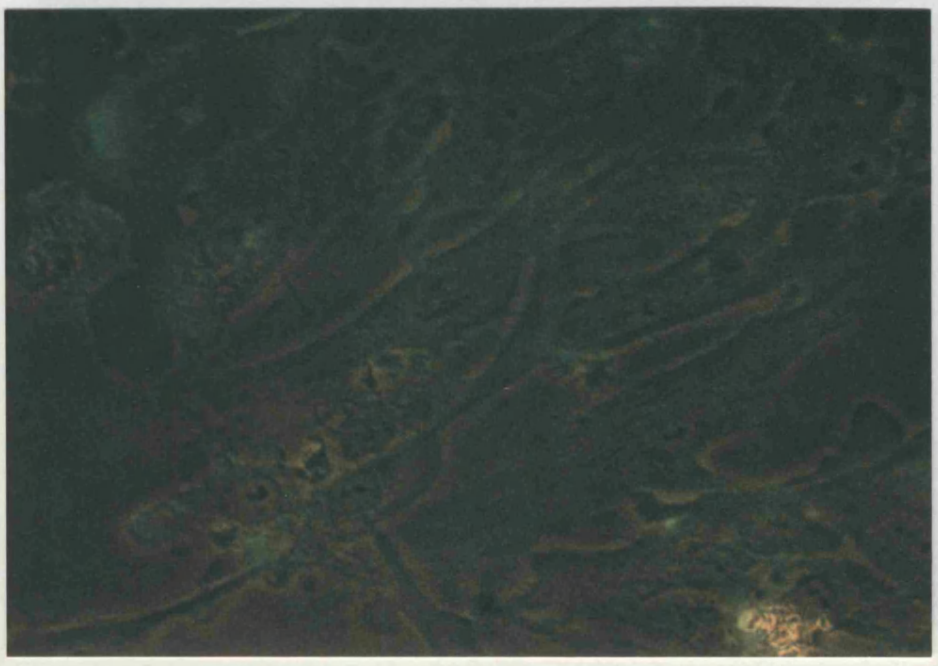


Figure 46. An area of epimerase deficient fibroblast line ED1 under phase contrast and combined UV and phase contract illumination.

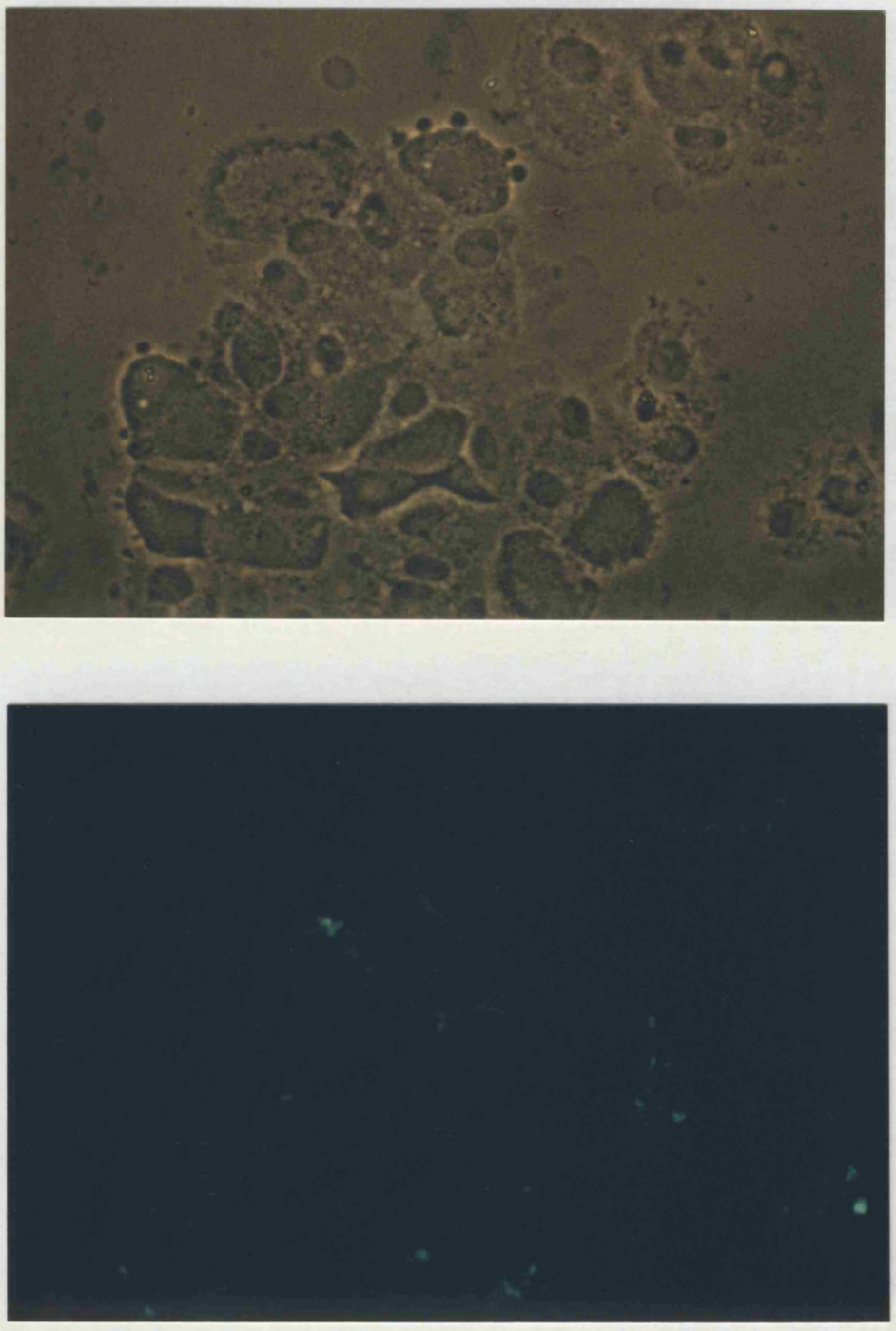


Figure 47. An area of wild type CHO cells under phase contrast and UV illumination.

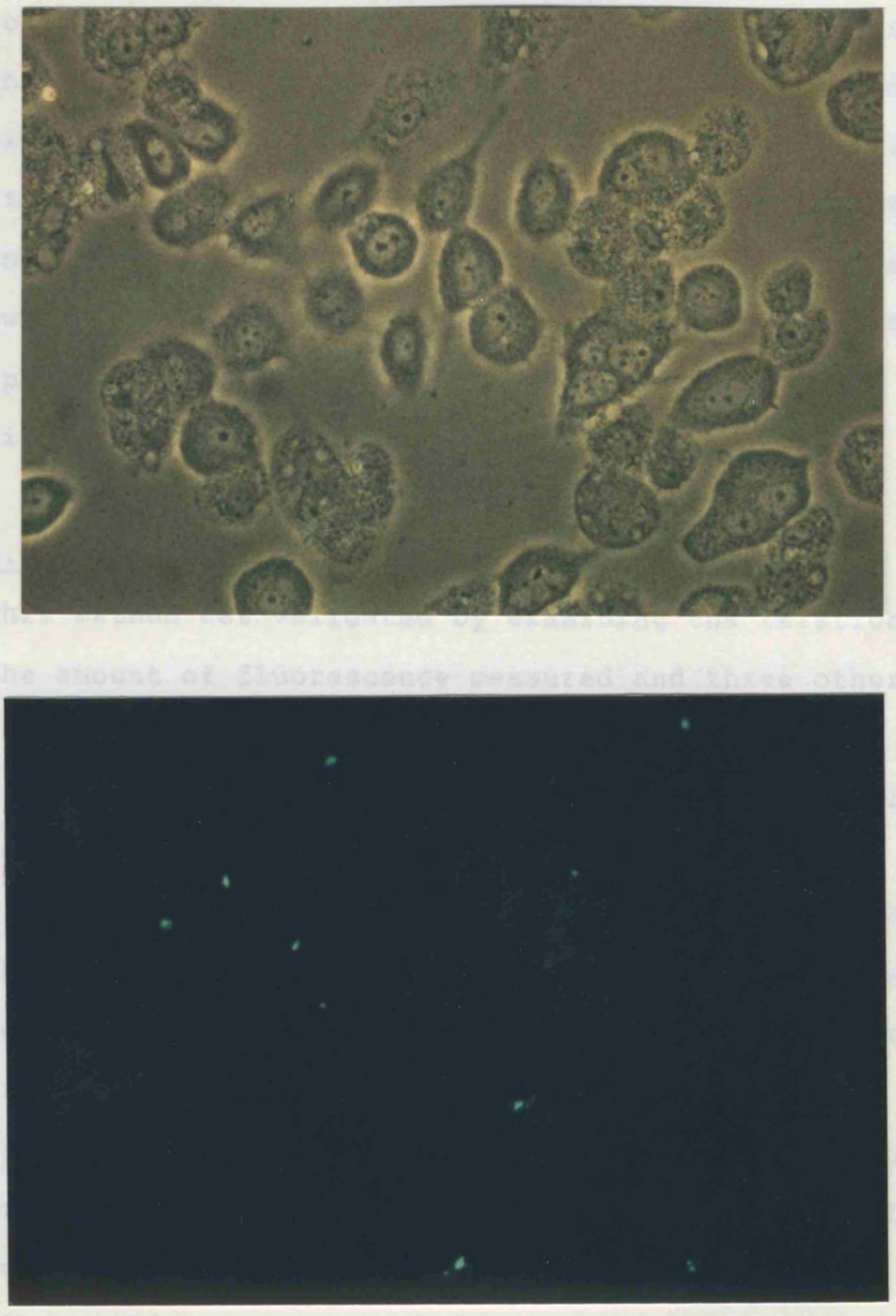


Figure 48. An area of epimerase deficient CHO cells under phase contrast and UV illumination.

In particular, the epimerase deficient fibroblasts appeared to stain in a similar manner to normal control fibroblasts. In contrast, epimerase deficient CHO cells appeared to stain significantly less brightly than wild type CHO cells. Figures 41-48 show photographs of cells under phase contrast and UV illumination. Representative examples are shown of two normal control, one transferase deficient and the epimerase deficient fibroblast lines, and both CHO cell lines.

4.5.4 Quantitation of Fluorescence.

This method was validated by examining the relation between the amount of fluorescence measured and three other variables: the number of cells present, the amount of protein present, and the amount of fluorescently labelled lectin added.

Figure 49 shows the amount of fluorescence measured against the cell count for a control fibroblast line and figure 50 for a CHO line. A linear relationship is evident from both graphs.

Figure 51 and 52 show fluorescence against the amount of protein present for control fibroblast and CHO cell lines. Once again a linear relationship is evident.

Figures 53 and 54 show fluorescence per unit protein as a function of lectin concentration for two control fibroblast lines, and figure 55 for a CHO line. The fibroblasts show an initial positive slope, which levels out suggesting that if at least 1 ml of lectin solution is added per well then the

amount of fluorescence detected is independant of the amount of lectin added.

The conditions derived as a result of the above experiments were used to quantitate the amount of fluorescence per unit protein for a number of cell lines.

These are shown in Table 7.

Figure 49. Fluorescence versus Cell Number for Fibroblasts

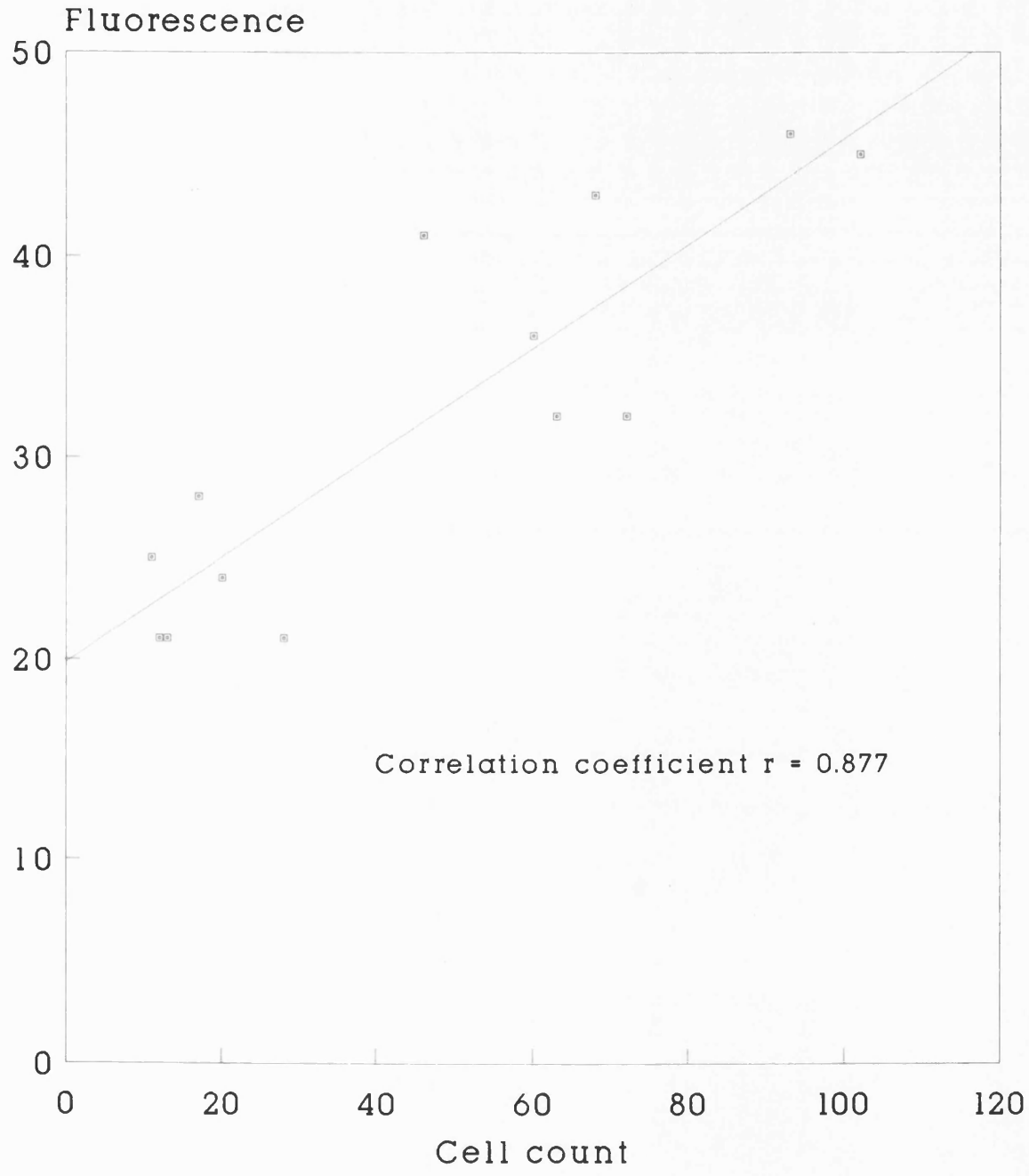


Figure 49. Fibroblasts were grown to a range of densities in multiwell plates. Wells were washed and one ml of a FITC solution (20 micrograms lectin protein per ml) in PBS was added and plates incubated for 30 mins. Wells were washed, cells trypsinised and counted, then dissolved in 1 ml of 0.1M NaOH and the fluorescence determined in a Shimadzu fluorimeter.

Figure 50. Fluorescence versus Cell Number for CHO Cells

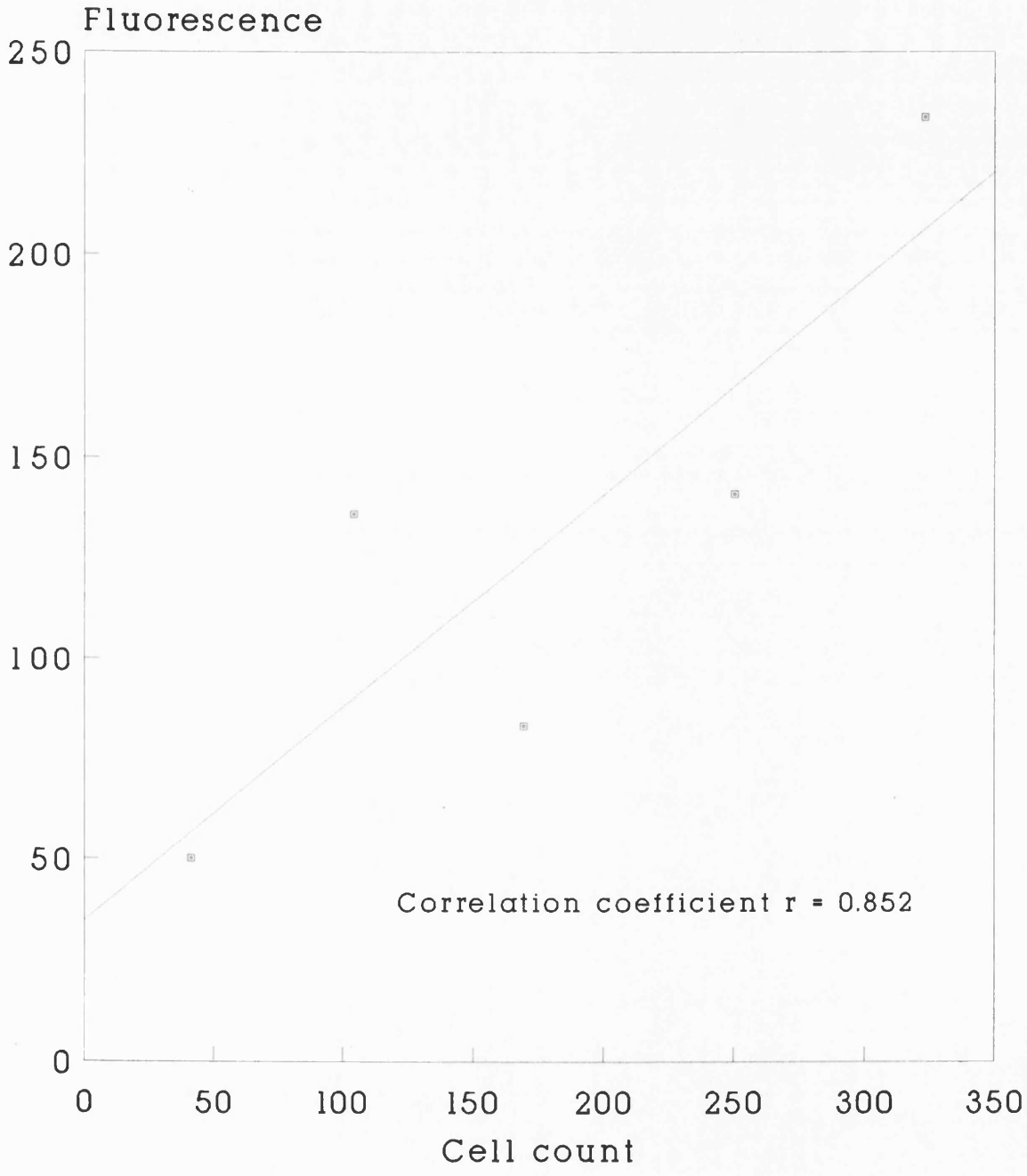


Figure 50. The experiment described in figure 49 was repeated for CHO cells.

Figure 51. Fluorescence versus Protein for Fibroblasts

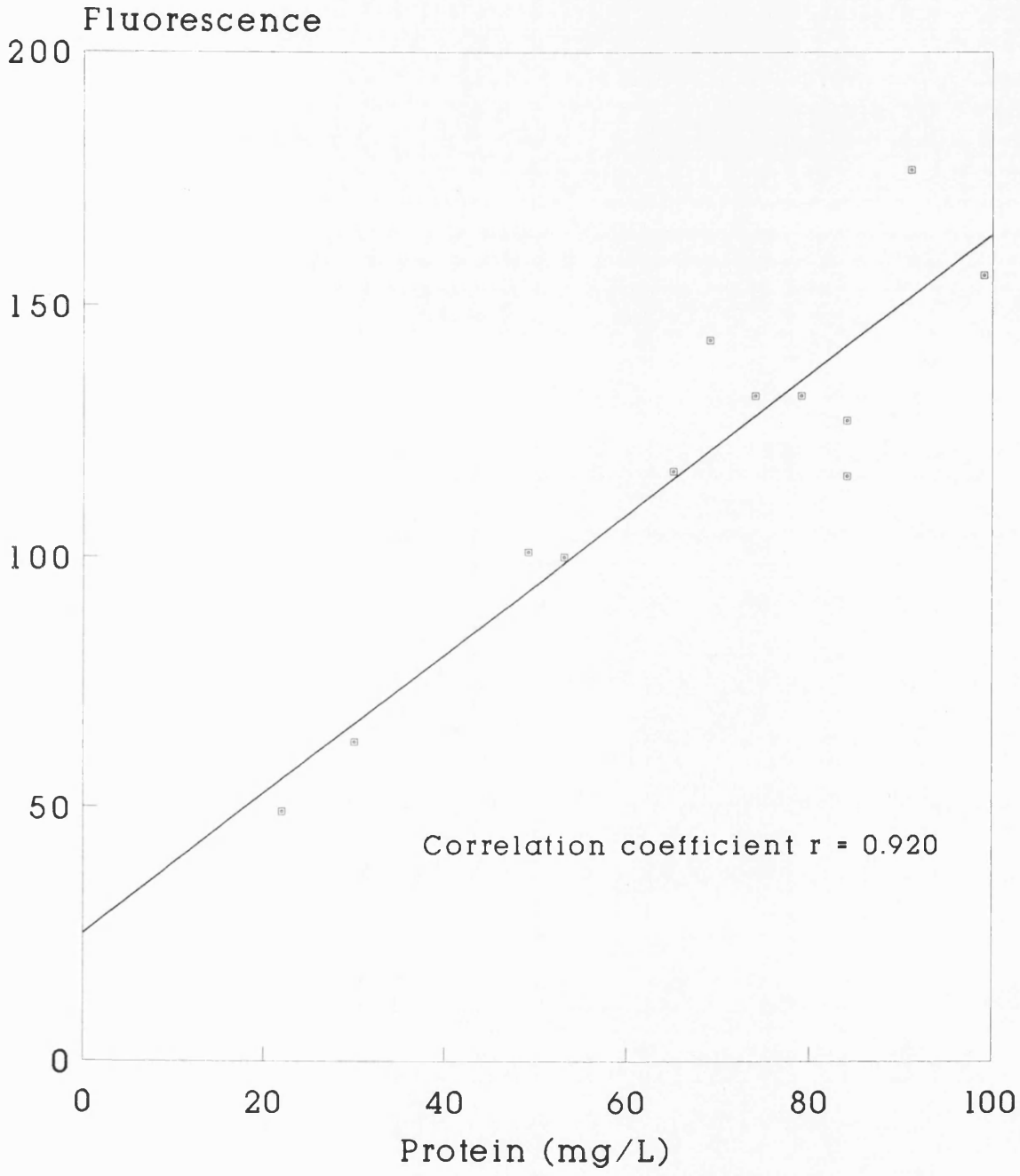


Figure 51. Fibroblasts from control line were grown to a range of densities in multiwell plates and stained with FITC lectin as described in figure 49. Wells were washed with PBS and the cell layer dissolved in 0.1M NaOH. Of this 400 µl were used for protein estimation by the Coumassie Brilliant Blue method and the remainder for quantitation of fluorescence.

Figure 52. Fluorescence versus Protein for CHO Cells

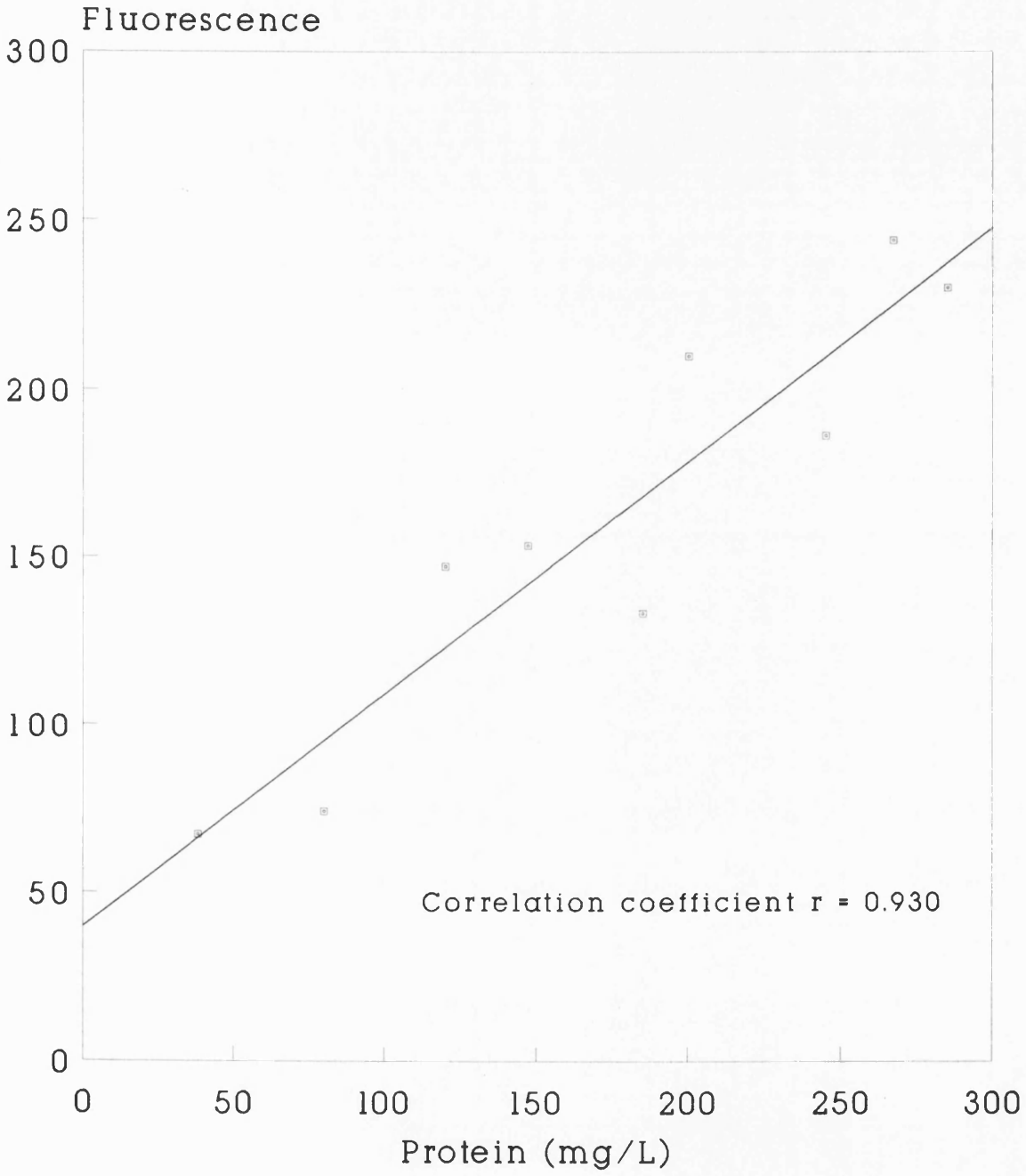


Figure 52. The experiment described in figure 51 was repeated using CHO cells.

Figure 53. Fluorescence per Unit Protein versus Amount of Lectin - Normal Control Fibroblast Line NC8

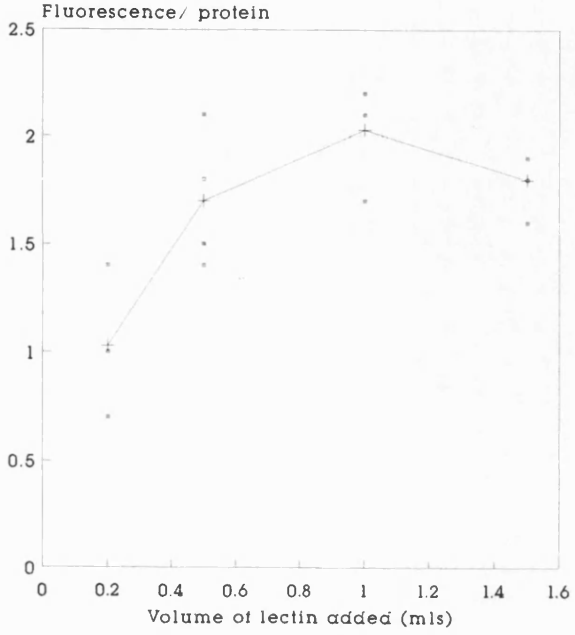


Figure 54. Fluorescence per Unit Protein versus Amount of Lectin - Normal Control Fibroblast Line NC14

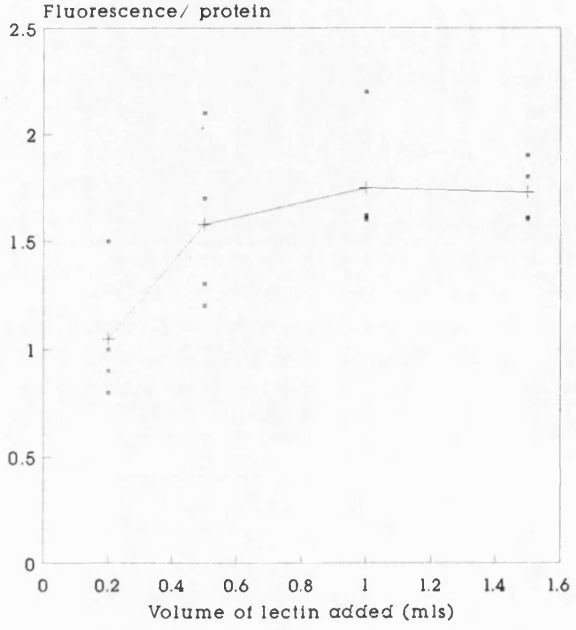
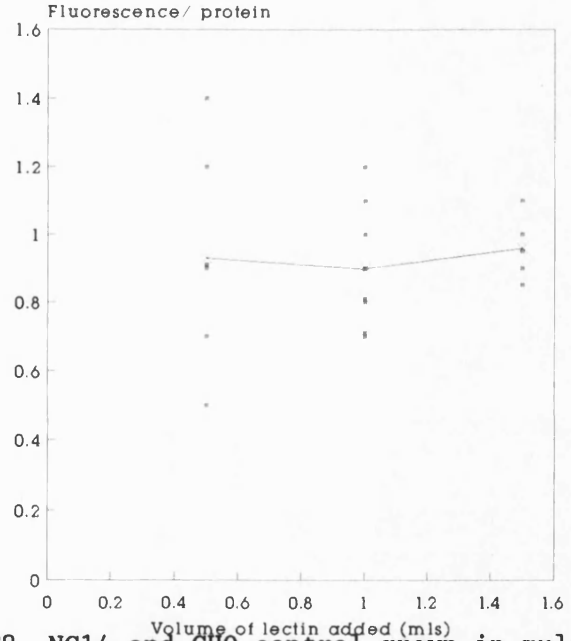


Figure 55. Fluorescence per Unit Protein versus Amount of Lectin - CHO Cells



Cells from lines NC8, NC14 and CHO control grown in multiwell plates to a range of densities. Various amounts (0.2 to 1.5 mls) of a solution of FITC lectin in PBS (20 µg lectin protein per ml) were added to the wells and plates incubated for 30 mins. Wells washed copiously and cell layer dissolved in 0.1 M NaOH. This was used for estimation of protein and quantitation of fluorescence. Results were plotted as fluorescence per unit protein vs. amount of lectin added to show whether the level of binding was dependent on the amount of lectin added.

Cell line	Fluorescence/protein	n	SD
NC2	0.52	8	0.06
NC9	1.70	6	0.18
NC10	0.83	7	0.15
ED1	0.81	8	0.10
TD1	1.48	8	0.35
TD2	1.46	6	0.31
TD3	1.33	6	0.30
CHO clone	0.64	12	0.11
CHO wild	0.80	10	0.16

Table 7. Fluorescence per unit protein. This quantitates the binding of fluorescein linked ricin lectin by intact cells. n = number of samples tested. SD = standard deviation.

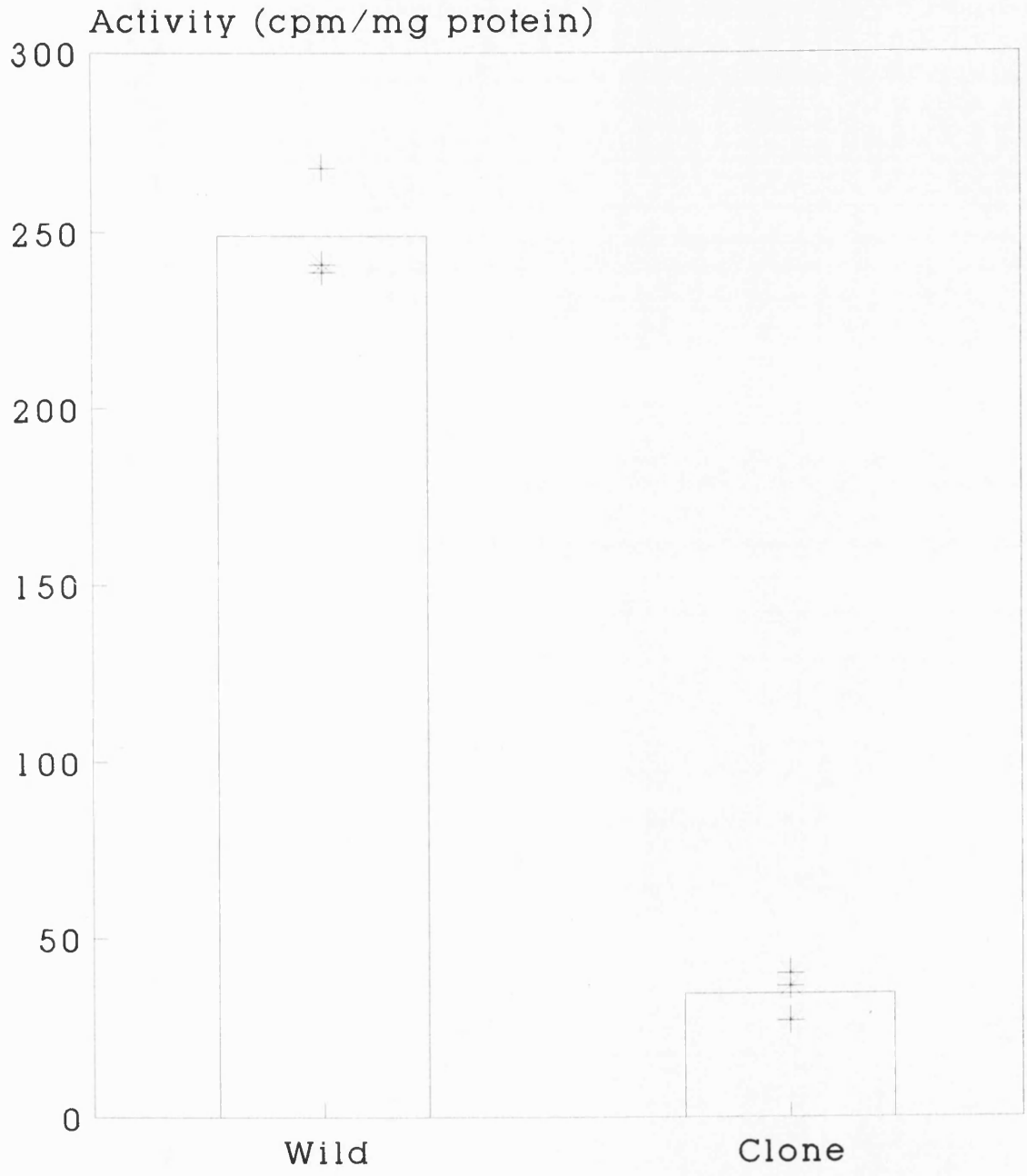
4.5.5 Carbon-14 CO_2 from labelled Galactose.

Initial experiments were carried out with the CHO cell lines. The results are shown in Table 8 and graphically in figure 56. The incubation period in this experiment was 24 hrs.

Cell line	Counts/mg protein/min	Mean
CHO wild	268 241 239	249
CHO clone	36.6 40.2 27.0	34.6

Table 8

Figure 56. Production of Radiolabelled CO2 from Galactose in CHO Cells



Erlenmyer flasks were prepared containing sub-confluent monolayers of CHO wild and clone cells. A paper disc soaked in concentrated NaOH was placed in the cap and the medium replaced with one supplemented with 0.1 µCi/ml of carbon-14 labelled galactose. Flasks were incubated for 6 hrs after which the carbon-14 CO_2 produced was estimated by beta-counting the discs in 2 mls of cocktail T scintillant, and the protein per flasks measured by the method of Folin and Ciocalteau. Three observations are shown with bar height equal to the mean.

These results show a level of activity 7.2 times greater in wild type cells, but they also show a significant and reproducible level of activity in the CHO clone cell line. The maximum percentage conversion of the labelled substrate was 0.3% for CHO wild cells and 0.025% for CHO clone cells. This experiment was also attempted with three normal control fibroblast lines. The maximum percentage conversion obtained with fibroblasts was 0.025% of total activity.

4.5.6 Section 5: Galactose in Cells: Discussion

All previous work on the abnormalities in human cells with disorders of galactose metabolism had concentrated on measuring levels of metabolites, rates of CO_2 production or levels of incorporation from labelled galactose (Xu 1989a, Kaufmann 1989) rather than assessing the levels and structures of the end points of incorporation such as glycoproteins, as is examined in this section.

Similar work had previously been carried out on epimerase deficient salmonella (Nikaido 1960 Fukosawa 1960). These were found to contain no galactose in their cell walls. It was not known whether this occurred in human or CHO cell lines. Xu (1989a) showed that incorporation of labelled galactose into TCA insoluble material was reduced in transferase deficient cell lines and hypothesised that some of the complications of galactosaemia are due to interference with the synthesis of galactose containing glycoproteins and glycolipids.

The experiments on the gas chromatography of carbohydrates from hydrolysed cells answers some of these questions. Firstly, both epimerase deficient lines contained galactose,

and in amounts not significantly different from controls. Thus they do not substitute glucose in the manner of epimerase deficient salmonellae.

Secondly, transferase deficient cells contain significantly lower levels of the ratio of galactose to mannose, and of sialic acid plus galactose to mannose than control fibroblasts. Sialic acid is found as a terminal residue on galactosyl chains. It had formerly been assumed that in the absence of transferase, the alternative pathway from glucose-1-phosphate via epimerase could maintain sufficient UDP gal for the synthesis of glycoproteins. The demonstration of low UDP gal levels in galactosaemic patients together with these results suggest that a disorder of galactosylation might play a part in the pathogenisis of galactosaemia, particularly in the long term complications of this condition. The ovary is a tissue particularly rich in galactose containing glycoproteins (Kaufmann 1988) and the profound ovarian dysfunction seen in female galactosaemics would appear to conform to this hypothesis. These results have been published (Dobbie 1990).

The results of the Western blot confirm the above observations that epimerase deficient cells contain galactose, using a completely different experimental technique and detection system. Cell line ED1 shows a similar pattern to controls suggesting that glycoprotein structures are not disordered, but at a slightly reduced density. This is in accord with the GC results which showed a lower total galactose level in line ED1. Transferase deficient cells also appear similar to controls; it therefore seems likely that the reduction found

by gas chromatography is spread across a wide range of glycoproteins. In the CHO lines, the epimerase deficient clone shows a different pattern from the control line, and as with fibroblasts the total galactose level by GC was lower in the clone. It is possible that this disordered pattern could be a result of the epimerase deficiency in the CHO cell lines being more severe than in the fibroblast line.

The observation and photography of FITC-linked lectin once again confirmed the presence of galactose on the cell membranes of all cell lines studied. The diffuse staining all over the cell surface is typical of specific lectin binding (Brachet 1985) and was completely suppressed in all cases by the addition of galactose. Because some subjective differences in the degree of staining were observed, a method of quantitating the degree of fluorescence was devised. This consisted of solubilising the cells and measuring the degree of fluorescence. Trypsin has been shown to alter the number of lectin receptors on cell surfaces (Sharon 1975) so it was not used in these experiments, except for the validation that fluorescence was a function of cell number where its use was unavoidable. Fluorescence was shown to be linearly related to cell number for fibroblasts and CHO cells (figures 49 and 50) and protein (figures 51 and 52), and unrelated to the volume of lectin added above 1 ml. The results of quantitation show that binding to the CHO clone is significantly lower than the wild type, and that transferase deficient fibroblasts are significantly lower than controls. These observations confirm the results found by gas chromatography.

The final experiments in this section concerned the oxidation of radiolabelled galactose to CO_2. Experiments on the CHO lines (table 8) showed a seven-fold difference in the rates of CO_2 production. There was however, a small but significant rate of oxidation by the CHO clone which has been described as having zero epimerase activity (Kingsley 1986). The production detected could result from residual enzyme activity, metabolisable impurities in the radio-label (eg labelled glucose) or metabolism via alternative pathways which have been shown to exist (Cuatrecasas 1966). It was therefore concluded that this was not an experimental technique whereby low levels of activity in the epimerase deficient fibroblast line could be detected.

4.6 Conclusions and Further Work

4.6.1 Conclusions

Cell Culture

It is possible to prepare media which contains no measurable free galactose. The amount of any free galactose which is present can be further reduced by dialysis and the resulting medium can be used to culture cells reliably.

The presence of combined galactose in foetal calf serum proteins was confirmed. Galactoproteins with exposed galactosyl residues may be partially removed with a solid phase linked lectin column. Since glycoproteins which contain unexposed galactosyl residues will not be removed by this technique it is not suitable for preparing a medium free of any combined galactose.

Transferase Deficient Cell Lines

By all the parameters assessed, transferase deficient cells will grow in normal growth medium at a rate similar to control lines over the time scale investigated.

The most significant finding in this work is that transferase deficient cells grown in normal media have a different balance of carbohydrates in their glycoproteins. In particular, galactose is significantly reduced. It has always previously been assumed that in the absence of transferase adequate levels of UDPgal could be maintained by its synthesis from glucose-1-phosphate. The demonstration in this work of abberant glycosylation in vitro suggests that the formation of defective glycoproteins in vivo may play a part in the pathogenesis of galactosaemia, particularly the

long-term complications of the condition. Abnormally glycosylated glycoproteins have now been found in vivo by Spaapen et al (1992). Two untreated female galactosaemic infants had transferrins in their sera which showed a pattern on isoelectric focusing suggestive of reduced glycosylation. This result supports the conclusions of this thesis and confirms its implications for metabolism in vivo.

Abnormal nucleotide metabolism in transferase deficient cells was confirmed in the experiments showing raised uptake of uridine and thymidine.

Recent work by Berry (1992) has shown that in vivo, galactosaemics have an increased ratio of UDP glucose to UDP glacatose compared with controls but they attribute this to dietary restriction of galactose, since a smaller increase in the ratio was also observed in subjects with other inherited disorders who were on milk-restricted diets. These observations are compatable with the results in this thesis, since the cells in which reduced amounts of galactosylation were found were grown in medium with no measurable galactose, i.e. they were on a "galactose free diet".

Epimerase Deficient Cell Lines

Epimerase deficiency had previously been considered likely to be a lethal mutation. However, both epimerase deficient fibroblasts and CHO cells were observed to grow in culture. There are three possible explanations for this:

1) **The alternative sugar:** epimerase deficient salmonellae substitute glucose for galactose in their cell wall glycoproteins: possibly CHO cells and fibroblasts do the same. It may be thought surprising that even a simple prokaryote can remain viable after

such a significant change: the much more sophisticated mammalian cells relies even more heavily on the exact stereochemistry of their constituent molecules. It is therefore theoretically unlikely that this can be an explanation for the growth of epimerase deficient cells. This hypothesis was experimentally disproved by the detection of significant amounts of galactose in the walls of both these cell lines.

2) **Abstraction:** cells incapable of synthesising galactose may abstract it from their growth medium, or they may conceivably abstract an enzyme capable of synthesising it. One of the objects of these experiments was to prepare a culture medium as low in galactose as possible. Whilst uncombined galactose was virtually eliminated, it was not feasible to produce a medium free of combined galactose. The required enzyme was not detected in the culture medium. Since the epimerase deficient salmonellae were not able to acquire sufficient galactose from their media it seems unlikely that fibroblasts or CHO cells could. However, it is not possible to rigorously exclude this explanation.

3) **Residual Activity:** Possibly these epimerase deficient cells grow because they are not truly epimerase deficient. It is impossible to prove that no activity exists in these cells, only that there is less than can be measured experimentally. Indeed many inherited enzyme deficiencies are caused by the production of a protein with a single amino acid substitution, usually in the active site. In transferase deficiency a protein immunologically identical to the missing enzyme but without measurable activity is produced. It is thus reasonable to

hypothesise that epimerase deficient cells grow because an altered enzyme is synthesised which has too little activity to be measured, sufficient to supply the cell with galactose for its structural requirements, but not enough to break down a galactose load, leaving the cells vulnerable to galactose toxicity.

It is therefore not possible to distinguish between possibilities 2 and 3, i.e. residual activity or abstraction from a low galactose environment on the basis of the experimental results contained in this work.

However, since Gillett (1985) found 11% of control levels of epimerase activity in a liver biopsy from the same index case that provided the fibroblast line, it is likely that in vivo at least residual activity is present in sufficient levels to provide adequate galactose for structural requirements.

The most important implications of this work are those for the treatment of epimerase deficiency in vivo. Although it is not possible to say precisely why, it is clear from the various assessments of cell growth that epimerase deficient cells grew adequately without any galactose supplementation but where a small amount of combined galactose was available. Sources of galactose-containing glycoproteins similar to those in foetal calf serum exists in meat and vegetable proteins in the human diet. It was also observed that epimerase deficient cells grew no better when supplemented with galactose.

All this suggests that the need for galactose supplementation in vivo for epimerase deficient subjects must be questioned.

However, before therapeutic recommendations are made it is vital to remember the limitations of the cultured cell as a model; the conclusions above apply to fibroblasts. Other classes of cells

such as neurones and oocytes are richer in galactoproteins; the effect on these cell types of the absence of galactose supplementation is not known.

A final implication of this work concerns the papers from the laboratory of Krieger (e.g. Kingsley DM and Krieger M and Holton JB 1986; Kingsley DM, Kozarshy KF, Hobbie L and Krieger M 1986). In these the authors describe how an amphotericin containing medium selects a population of CHO cells which are deficient in low density lypoprotein (LDL) receptors, which may be used as a model for familial hypercholesterolaemia. These cells were found to be epimerase deficient, and in fact were the CHO clone used in this work. Krieger states "complete deficiency of epimerase.. disrupts the synthesis of many glycoproteins and glycolipids. As a consequence, the hamster cells synthesise abnormal forms of the LDL receptor, a glycoprotein". The results described in this thesis show by three different analytical techniques that galactose is present in significant quantities in the cell walls of this clone of CHO cells when grown as described. Krieger (1983) states that high concentrations of serum may restore LDL receptor activity but gives no further details and refers only to "unpublished data" and gives no figure for the percentage of foetal calf serum used. In another paper in this series (Kingsley et al 1986) 5% of foetal calf serum is used, the same as in the experiments described here. The results in this thesis indicate that the lack of LDL receptors in this clone of CHO cells does not result from the absence of galactose, a structural component of these receptors.

4.6.2 Suggestions for further work

No further experiments with radiolabelled galactose are advocated, either for measuring production of radiolabelled CO_2 or inclusion in acid precipitable fractions of cell homogenetics. Such experiments would be hampered by the limited purity of commercial radiolabels and metabolism by the minor pathway of galactose to xylulose. They are therefore likely to be uninterpretable.

Further experiments suggested by the conclusions of this work which are likely to produce meaningful results include the following:

1) If the epimerase deficient salmonella used by Nikaido (1961) which were found not to have galactose in their cell walls could be obtained they could be grown in the media used in these experiments and analysed for galactose. Absence of galactose would support the contention that there is insignificant galactose available in this medium.

2) Transferase deficient cells were shown to have abnormal glycoproteins, but in all growth experiments were found to have growth rates similar to control cells. This suggests that either the cells were not handicapped by their reduced levels of galactose or that the experiments were not conducted over a sufficiently long timescale to observe any effect. This is concordant with the observation that in humans, once on a galactose free diet galactosaemics develop in an ostensibly normal fashion until long term sequellae become apparent, such as infertility. It would therefore be appropriate to examine cells over a longer timescale than 7 days to see if differences in cell growth become apparent.

3) The implications for the therapy in vivo of epimerase deficiency warrant further consideration. It would be important to examine whether the observation that fibroblasts need no galactose supplementation applies to other cell types. The only other easily obtained culturable cell type would be lymphocytes. These could be subjected to the same analysis as fibroblasts. An alternative consideration of the problem would be to analyse tissue or blood samples from affected subjects. The presence of a normal level of galactosylation in proteins in vivo of a subject on a galactose free diet would provide the best possible evidence that galactose supplementation would be of no benefit to subjects affected by epimerase deficiency.

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